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A CYTOLOGICAL STUDY OF HETEROTHALLISM IN PUCCINIA TRITICINA[1]

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INTRODUCTION

The announcement by Craigie in 1927 of heterothallism in the rusts has opened up a new field of research of both theoretic and practical interest. The first paper (*7*)[3] in which experimental proof was given of heterothallism in *Puccinia helianthi* Schw. was soon followed by a second paper (*8, p. 765*), presenting similar proof for *P. graminis* Pers. Here, too, evidence was given that the pycnia or spermogonia are not,

> as many botanists have supposed, male conceptacles producing non-functional spermatia, but are active organs having a non-male function which they carry out through the agency of flies. * * * the mycelium, pycnia, and pycnospores of some of the pustules were (+) in sex, whereas the mycelium, pycnia, and pycnospores of other pustules were (−) in sex.

When a monosporidial pustule is kept isolated it produces pycniospores (haploid) but no aeciospores (diploid). But when the nectar (exudate containing the spores) of a (+) infection is transferred to the nectar of a (−) infection (or vice versa), aeciospores form. The pycniospores (spermatia) bring about the change from the haploid to the diploid phase.

In a third paper by Craigie (*9*) additional data are given in support of the above conclusions, and field observations are recorded which indicate that *Puccinia coronata* Cda., *P. pringsheimiana* Kleb., and *Gymnosporangium* sp. also are heterothallic.

While these studies established the fact of heterothallism in *Puccinia graminis*, nothing was yet known of the actual process taking place in the host plant. However, in 1929 Hanna (*12*) published a preliminary account of studies in this field. He found that in the pustule of monosporidial origin the mycelium and pycnia are uninucleate and that such an infection produces, near the lower epidermis of the leaf, sterile wefts, "which appear to be crescent-shaped in trans-

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[3] Reference is made by number (italic) to Literature Cited, p. 753.

verse sections of the leaf" and which "are evidently haploid rudiments of aecial cups waiting to be stimulated into further developmental activity." He observed that pycniospores germinate, the largest germ tube seen being 15μ long, and that in the infection to which pycniospores of opposite sex have been transferred, the sporophyte generation appears and open aecia develop. He found that the first binucleate cells are formed in the base of the aecium by the fusion of cells in pairs in the manner described by Christman (*6*), the fusion cell becoming the basal cell of a spore chain.

In a paper covering the same ground the writer (*1*) agrees in the main with Hanna's observations but finds evidence that the sporophyte generation begins earlier. Mycelial cells with more than one nucleus have been found at the pycnium, in the mycelium, and in the young aecium before the sporogenous layer was differentiated. Fusion of cells at the base of the aecium to form 2-legged basal cells has not been seen.

The fact that in certain rusts the two cells that fuse to initiate the diploid generation come of different parents suggests the possibility that physiologic forms and varieties of those rusts may hybridize on the aecial host and that new physiologic forms may arise from such crosses. Experiments along this line are in progress in several laboratories and preliminary reports of three of these (*16*, *18*, *20*) have been published.

Waterhouse (*20*) in Australia crossed two physiologic forms of *Puccinia graminis tritici* Eriks. and Henn. on the barberry and obtained from the cross two physiologic forms new to that continent.

According to Stakman, Levine, and Cotter (*18*), crosses between *Puccinia graminis tritici* and *P. graminis agrostidis* Eriks. may result in a number of physiologic forms of *tritici*, some previously known and others new to science. From one cross eight physiologic forms were isolated, apparently from the spores of a single aecial cup. From crosses between *P. graminis tritici* and *P. graminis secalis* Eriks. and Henn., the parental forms were recovered, together with other physiologic forms, some of which were new. In long-continued uredinial cultures of certain physiologic forms of *tritici* new physiologic forms have appeared by mutation.

According to Newton, Johnson, and Brown (*16*), the selfing of a physiologic form may result in several forms, some known previously and others new to science. Only one of the physiologic forms used in their experiment proved homozygous. They found that when 2 physiologic forms were crossed, in a few cases a parent form reappeared, but more commonly a different form appeared, and that when the mixed exudate of 8 physiologic forms was used in crosses, 17 forms appeared, of which 7 were new. Spores from each aecial cup in this experiment were cultured separately; in 95 per cent of the cases only 1 physiologic form was isolated from each cup.

Andrus (*3*) in a paper [4] presents an account of the gametophytic development and fertilization of *Uromyces appendiculatus* (Pers.) Fries and other rusts. Andrus found that in *U. appendiculatus* the sporidium gives rise to a haploid mycelium that bears spermogonia and trichogynes. He states that the gametophytic hyphae which

[4] A paper delivered before the mycological section of the Botanical Society of America in December, 1930, and made available to the present writer through the courtesy of its author. A report of this investigation (*3*) has appeared since the present paper was prepared.

function as trichogynes are much branched and highly septate organs, having their terminus at the epidermis of the host leaf where they project through stomata or between epidermal cells and make contact with spermatia. Andrus found that the rust is heterothallic—an isolated infection remains haploid. When spermatia from one infection are transferred to a different infection and applied to the leaf surface, the nuclei of the spermatia enter the trichogynes and pass to their base, where they become associated with the native nuclei to form the beginning of the sporophyte generation. By proliferation of these cells, and sometimes by supplementary fusions, a sporogenous layer of binucleate cells is formed from which spring the spore chains of diploid cells. The Christman theory of fertilization (*6*) in rusts is held to be no longer tenable.

Much cytologic work on the aecial generation of rusts (*1*) was published prior to the discovery of heterothallism in this group. In such investigations stages of the isolated sterile infection, if accidentally encountered in the material studied, would either be incorporated with the rest as a part of the story of development of the fertile infection or be cast out as "pathologic." Moreover, previously accepted observations as to the mode of origin of the sporophyte generation in the aecium are now in need of a critical repetition in the light of recent discoveries. In order to further the knowledge of the microscopic details of heterothallism, a cytologic study of *Puccinia triticina* Eriks. was undertaken by the writer.

MATERIALS AND METHODS

Spores of *Puccinia triticina* were obtained from E. B. Mains, H. B. Humphrey, Margaret Newton, and C. O. Johnston, and plants of *Thalictrum flavum* L. were obtained from E. B. Mains. Seed of another species of Thalictrum was purchased as *T. delavayi* Franch., but was probably *T. dipterocarpum* Franch. The plants proved susceptible to the rust.

The plants were grown in the greenhouse, and the experiments were carried out there. The straw bearing teliospores was placed in loose-mesh cloth bags. A part was kept in cold storage, and the rest was kept in the field, in a partly shaded spot on the ground. The latter method proved the more satisfactory.

In inoculating, watch crystals were filled with mud; bits of the rusted straw were soaked in rain water several hours and sprayed with an atomizer to stimulate germination, then pressed into the mud, with the spores exposed, and sprayed again. A bit of wet sphagnum was wrapped around the base of each plant. Tall glass tumblers with the bottoms removed and the sides lined with wet paper were placed over the plants and the mud-filled crystals used as lids. A layer of wet paper was folded down over the top and held in place with a rubber band. This placed the rusted straw directly above the leaves, and as the sporidia were formed and set free they fell on the leaves. The whole was placed under a greenhouse bench for 48 hours, then uncovered and replaced on the bench.

The inoculated plants were covered with tarlatan cages to exclude insects. These cages were effective in excluding flying insects, but did not keep out thrips or red spiders. Every effort was made to keep the plants free from these latter; but, despite precautions,

occasional infections were fertilized through their agency. The infections were studied and greenhouse records of them were kept.

Material was fixed daily for the first three weeks and at longer intervals from then on until the infections were 6 weeks old. The fixing fluids used were chrom-acetic formalin and Flemming's medium and weak solutions. Of these, Flemming's weak solution was most trustworthy. The material, after remaining 36 to 48 hours in the fixing fluid, was washed, dehydrated, and embedded in 50° paraffin. The principal stains used were safranine and methylene blue.

INVESTIGATIONS

ENTRANCE AND DEVELOPMENT OF MYCELIUM

After 24 hours in the inoculating chamber some of the teliospores had germinated, the promycelia had produced and freed sporidia which had fallen on the leaves, and some of the sporidia had then germinated and entered the epidermal cells of the leaves.

Plate 1, A, shows an epidermal cell with the young rust fungus from a 1-day infection.[5] The host cell is somewhat plasmolyzed. Flemming's medium solution, used in fixing this preparation, proved too strong for Thalictrum leaves. On the outer surface of the cell is the sporidium (*a*), which is only partly evacuated. Even in older preparations there is sometimes a remnant of the spore plasm left in the sporidium. The bulk of the sporidial content has entered to form the sacklike mass (*b*) within the epidermal cell. There are apparently six nuclei in this cell. A comparison with later stages (pl. 1, B and C) makes this seem doubtful, or at least exceptional. However, it would be expected that one or two nuclear divisions would take place before septation.

Plate 1, B, *a*, shows a newly septate primary hypha. It is slender, consisting of three uninucleate cells, and runs diagonally across the epidermal cell.

The primary hypha develops further, growing apically to form new cells, while the older cells become thicker and heavier and put forth branches. In Plate 1, C, drawn from a 2-day infection, is a 4-cell primary hypha (*a–d*) and the remnant of the original sporidium (*e*). The two older cells (*c* and *d*) have thickened and produced short branches. Their cytoplasm is vacuolate, much of it having flowed out into the branches, but the nuclei are still in the parent cells. The two younger cells (*a* and *b*) are still slender, dense, and unbranched.

In the later growth the primary hypha and its branches become heavier and somewhat tangled, and it is often difficult to trace their course. Plate 1, D_1 and D_2, shows successive sections of a 3-day

[5] For the sake of uniformity and clearness, the drawings are oriented in the plates as the tissues are in the leaf, i. e., having the tissues nearest the upper surface of the leaf uppermost in the drawing.

EXPLANATORY LEGEND FOR PLATE 1

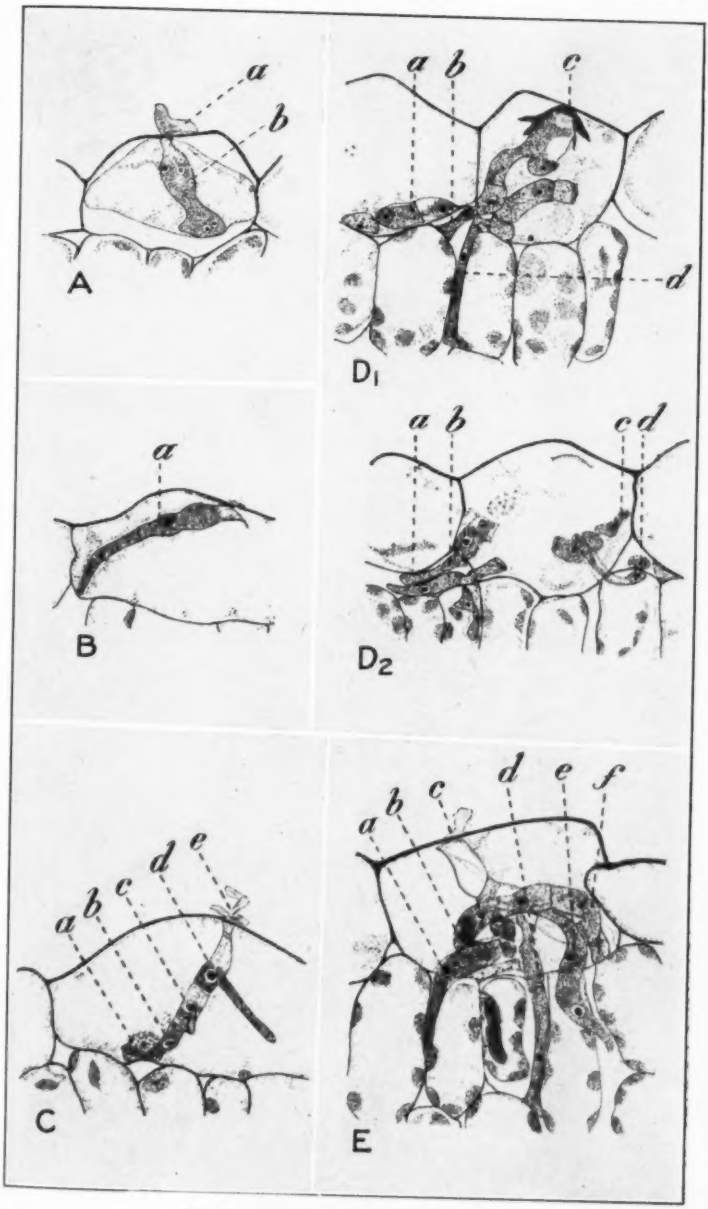
A.—One-day infection. The sporidium (*a*) has germinated and entered the epidermal cell at *b*. × 1,020.

B.—Two-day infection. Primary hypha (*a*) composed of three uninucleate cells. × 1,020.

C.—Four-cell primary hypha (*a–d*) and remnant of sporidial wall (*e*) from 2-day infection. The older cells (*c* and *d*) are pushing out branches. × 1,020.

D_1 and D_2.—Successive sections of a 3-day infection showing primary hypha (D_1, *c*, and D_2, *c*) and its branches (D_1, *a*, *b*, and *d*, and D_2, *a*, *b*, and *d*). × 1,020.

E.—Three-day infection. Primary hypha entered at *c*, grew to *f*, and doubled back to *b*. Branches at *a*, *d*, and *e*. × 1,020.



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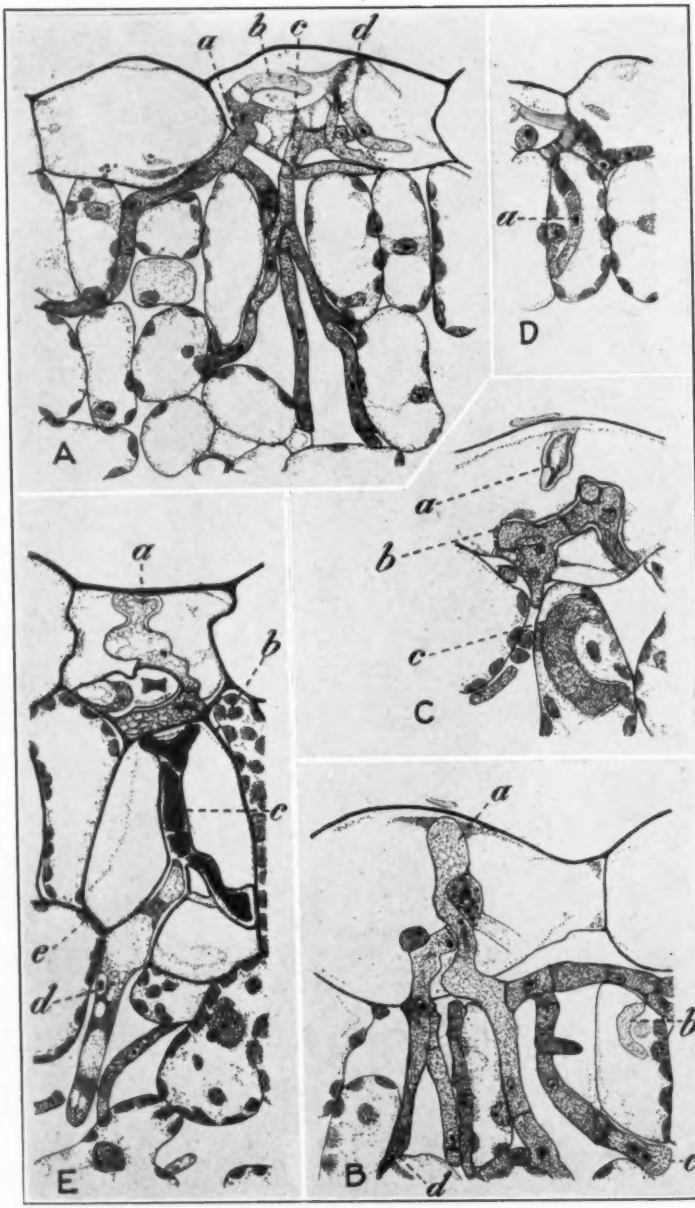

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infection. The primary hypha entered at D_1, *c*, swung to the left then curved back to the right, ending at D_2, *c*. Apparently, the terminal cell (D_2, *c*) is unbranched. Not less than 6 branches (and probably more) have formed and grown out of the host cell, 2 into other cells (pl. 1, D_1, *a*, and D_2, *d*), and 4 into intercellular spaces (pl. 1, D_1, *b* and *d*, and D_2, *a* and *b*).

In contrast to *Puccinia graminis* (*1*), these branches from the primary hypha do not make their exit from the epidermal cell through a small pore in the host cell wall, but from a relatively large hole often equal in diameter to the hypha. This is evident in Plate 1, D_1 and D_2, and shows still more clearly in Plate 1, E. Here (pl. 1, E) the rust entered at *c*, and the primary hypha swung to the right at *f*, then doubled back to *b*. It consists of 6 cells, of which the 4 older have branched and are now more or less evacuated, while the 2 younger are still unbranched. Three of the large vigorous branches are undiminished in diameter at the point of exit from the epidermal cell. (Pl. 1, E, *a*, *d*, and *e*.) These branches are making their way down between the palisade cells to the more open air spaces below.

Progress by the fourth day is shown in Plate 2, A. The primary hypha has entered at *d* and curved around to *b*. As before, the primary hypha itself does not leave the host cell, and its terminal cell (*b*) is unbranched. Branches at *a* and *c* have grown down into the host tissues, forming the mycelium. Ordinarily a cell of the primary hypha gives rise to only one branch. The cell at *d*, however, has formed two branches, neither of which is effective.

Once the mycelium is established, little or no further development occurs at the point of entry, and degenerative changes of the primary hypha set in. Plate 2, B, drawn from a 7-day infection, shows the primary hypha (*a*) breaking down, but a rich growth of branching hyphae radiates from it. This mycelium is haploid. The rapidly growing terminal cell of a hypha, however, may have two nuclei just before a septum divides it into two cells. (Pl. 2, B, *c*.) Rarely, there may be more than two. (Pl. 2, B, *d*.)

By the seventh day another change usually is noticeable. Plate 2, C, *a* and *b*, shows the primary hypha (divided in sectioning) coated by a layer of material staining like the host cell walls and possibly serving as a defense of the host against the intruder.

This sheath persists for a time after the primary hypha that it incloses has disintegrated, and becomes of value in later studies. Whether an infected area is of monosporidial or of multisporidial origin may be determined by the presence of one or of more than one sheath in the cells of the upper epidermis.

After leaving the epidermal cell the hyphae make their way down to the large air spaces of the spongy mesophyll. Ordinarily, they follow intercellular channels, passing through the natural spaces between palisade cells or, in case of need, forcing a passage by splitting

EXPLANATORY LEGEND FOR PLATE 2

A.—Four-day infection with primary hypha (*b* and *d*) and branches forming intercellular mycelium (*a* and *c*). × 1,020.

B.—Seven-day infection with primary hypha (*a*), mycelium (*c* and *d*), and haustorium (*b*). × 1,020.

C.—Seven-day infection with degenerating hypha (*a* and *b*) insheathed in cell-wall materials, and haustorium (*c*). × 1,020.

D.—Three-cell haustorium or hypha (*a*) in palisade cell of 4-day infection. × 1,020.

E.—Eleven-day infection with remnant of primary hypha (*a*) giving rise to the hypha *c*–*d*, which passes through the palisade cell *b*–*e*. × 1,020.

in two the wall between two palisade cells. (Pl. 1, D_1, *d*, and E, *a* and *d*.)

More rarely a hypha will attempt to grow through a palisade cell. Plate 2, D, *a*, shows a 3-cell hypha in a palisade cell. It is intermediate in character between a hypha and a haustorium and perhaps would not have grown further. In Plate 2, E (from an 11-day infection), however, the attempt to grow through a palisade cell has been successful. From the primary hypha (*a*), now disintegrated and misshapen, a hypha (*c*) entered the palisade cell (*b–e*), grew down through it and then out at *d*. The palisade cell and much of the hypha within it are dead.

The developing mycelium extracts food from the host cells by means of haustoria. These may be small, 1-cell, and unbranched, or they may be larger and more complicated. Examples of simpler haustoria are shown in Plate 2, C, *c*, and B, *b*, and in Plate 3, A, *c*, and D, *h*. Larger, branched haustoria consisting of more than one cell are shown in Plate 3, E, *a*, F, *a*, and G, *a*. In old age the haustoria, like the primary hyphae, often become insheathed in materials staining like host cell walls.

SPERMOGONIA AND RECEPTIVE HYPHAE

After six or seven days of vegetative growth, during which the mycelium spreads through the air spaces of the mesophyll, reproductive activity sets in. Hyphae grow to both the upper and the lower epidermis, giving rise there to spermogonia (pycnia) and to receptive hyphae.

When a spermogonium is to form, hyphae grow to the epidermis (upper or lower) and form a small compact group of dense, well-nourished cells in contact with the epidermal layer. Plate 3, A, shows an early stage of this process. Even at this early stage there is a beginning of organization, for hyphae at *a* and *d* are slanting up toward a common center at *b*.

Soon after this a more definite arrangement of hyphae is evident. In a median section through a young spermogonium from a 7-day infection (pl. 3, B), the hyphae growing in from all sides are definitely centered at *b*. The slender hyphae at *a* and *c*, close to the epidermis and nearly parallel to it, will become the paraphyses. The thick upright hyphae at *b* will become the buffer cells that lift the epidermis and resist its pressure.

Plate 3, C, shows a later stage of development. The buffer cells at *c* have become heavy upright columnar cells pressed against the epidermis. Their cytoplasm has taken on the open alveolar structure characteristic of buffer cells. Growing in between the buffer cells from the base and sides at *b*, *d*, and *e* are the first of the young spermatiophores that later will produce spermatia. At *a* and *f* are the young

EXPLANATORY LEGEND FOR PLATE 3

A.—Detail of 7-day infection showing beginning of formation of spermogonium at *a*, *b*, and *d*. Haustorium at *c*. × 640.

B.—Slightly older spermogonium from 7-day infection. Young buffer cells at *b*, and paraphyses at *a* and *c*. × 640.

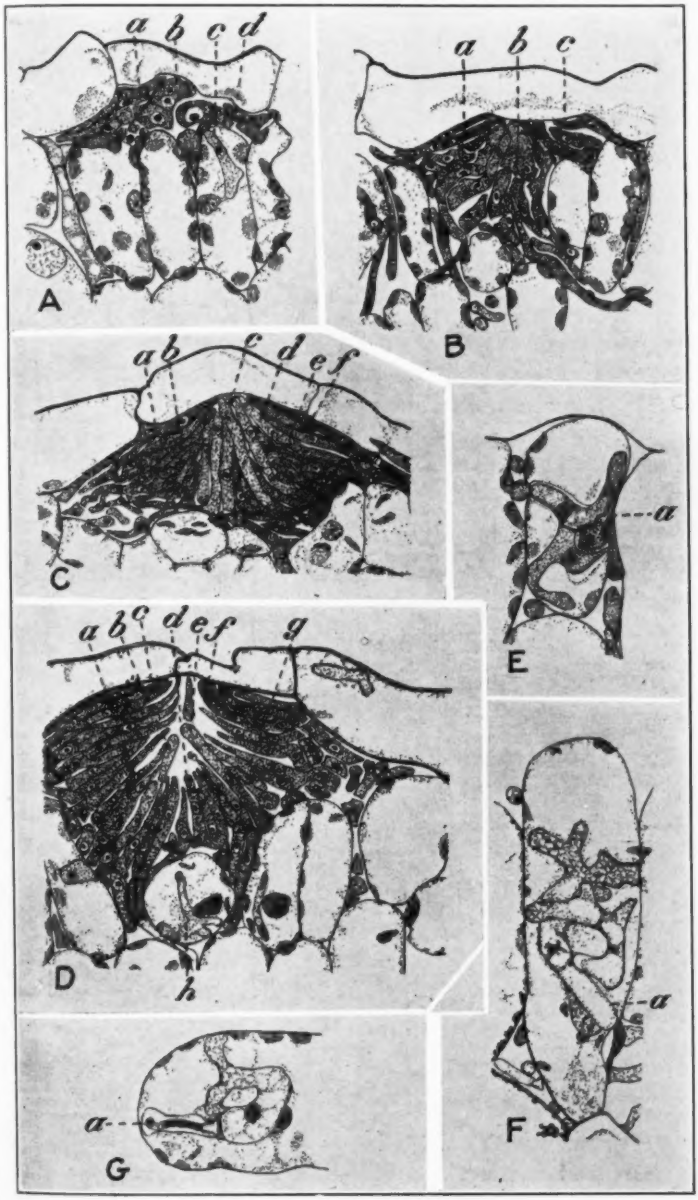
C.—Growing spermogonium with buffer cells at *c*, paraphyses at *a* and *f*, and spermatiophores at *b*, *d*, and *e*. × 640.

D.—Older spermogonium from 8-day infection with buffer cells at *b* and *e*, spermatiophores at *a*, *d*, and *g*, and paraphyses at *c* and *f*. Central cavity forming. Haustorium at *h*. × 640.

E.—Two-cell haustorium at *a* from 7-day infection. × 1,020.

F.—Much-branched haustorium (*a*) from 9-day infection. × 1,020.

G.—Decadent haustorium (*a*) from 9-day infection. × 1,020.



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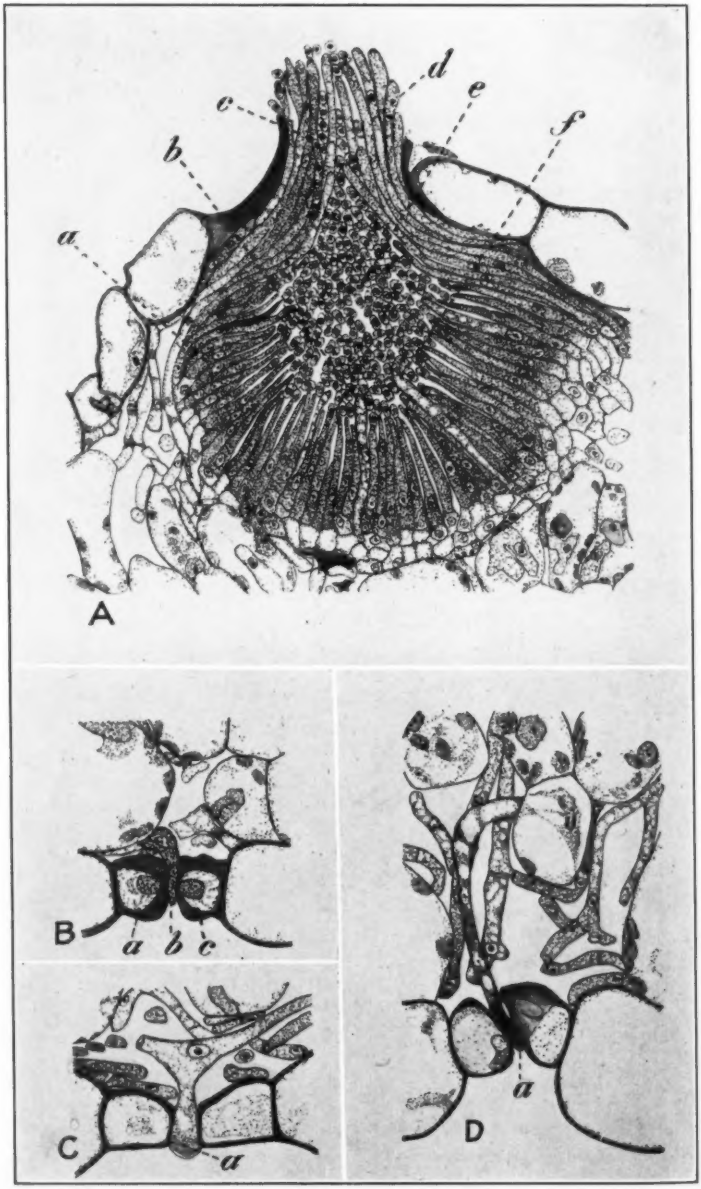

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paraphyses. All these hyphae, whether basal or lateral in origin, are focused on the central point (*c*).

A rapid increase in the number of spermatiophores follows. These are slender tapering cells that grow in from the base and side walls of the spermogonium. The interpolation of these new cells between the buffer cells (pl. 3, D) causes a rapid expansion of the spermogonium and arches it out into a spherical mass that crushes the adjoining host cells as it grows. As the spermogonium rounds out, a central cavity forms within it, lined by the tips of the spermatiophores (*a*, *d*, and *g*). The buffer cells (*b* and *e*), released from pressure against the epidermis, project into the central cavity. They produce no spores and soon wither and die.

Plate 4, A, shows a newly opened spermogonium from a 9-day infection. The paraphyses (*c* and *d*) have pierced the epidermis forming the ostiole and are growing out through the opening. The basal part of the spermogonium forms a hemispherical shell of radially arranged spermatiophores, among which is still to be found an occasional buffer cell (*b* and *f*). Spores formed by the spermatiophores fill the central cavity (*e*) and are moving out through the ostiole. The spores are exuded in a viscous liquid, the nectar, which has a distinctly flowerlike odor. The exudate is not abundant, but serves by its perfume to attract insects.

The first spermogonia usually mature and open seven or eight days after inoculation. The number of spermogonia is highly variable, however. In 9-day infections there may be as many as a dozen open spermogonia, or only two or three, or none whatever.

In *Puccinia triticina* the spermogonia are about equally distributed between the upper and lower surfaces of the leaf. A count shows that of 100 spermogonia in 11-day infections, 46 opened on the upper surface of the leaf and 54 on the lower.

While spermogonia are developing, certain hyphae are growing into stomatal apertures or forcing a passageway between epidermal cells of the leaf. Since these hyphae reach the surface of the leaf and serve to receive the spermatial nuclei, the terms "emergent hyphae" or "receptive hyphae" have been applied to them.

In the stomata, which in Thalictrum are on the lower surface of the leaf, hyphae of the fungus are to be found inserted into stomatal apertures. Plate 4, B, shows an early step in this process. A vigorous young wedge-shaped hypha (*b*) is pushing between the two guard cells (*a* and *c*) of the stoma. Whether such a hypha can force entrance into a closed stoma or must await its natural opening is not known; the latter seems probable.

Plate 4, D, shows another hypha. The stoma here is cut obliquely. In this instance several other hyphae are growing toward the same stoma. These hyphae, like the mycelium producing them, are composed of uninucleate cells. Plate 4, C, shows the further development of a cell in a stoma. The inserted hypha has grown larger and its broadened tip has reached the lower surface of the leaf.

EXPLANATORY LEGEND FOR PLATE 4

A.—Mature spermogonium from 9-day infection. Paraphyses (*c* and *d*) protruding through ostiole. Radially arranged spermatiophores (*a*) have formed spores filling central cavity (*e*). Buffer cells (*b* and *f*). × 640.

B.—Young receptive hypha (*b*) thrust into stoma (*a–c*); 9-day infection. × 1,020.

C. Older receptive hypha (*a*) in stoma of 11-day infection. × 1,020.

D.—Receptive hypha (*a*) in stoma of 9-day infection. × 1,020.

A less conspicuous formation of "emergent hyphae" occurs at the upper epidermis of the leaf. Plate 6, A, represents a detail from an 11-day infection showing the upper epidermis (*a*) and the palisade layer (*c*) beneath it. Between the two cell layers at *d* is a scant subepidermal mycelial growth, and from this at *b* a hypha has grown up between the epidermal cells to the upper surface of the leaf. It has not pierced the cuticle at the outer epidermal wall.

A few of these hyphae emerge at the upper and lower surfaces when infections are 7 days old and many more by the eighth or ninth day, but the development is not uniform. A survey of 9-day infections shows that an infection which bears numerous spermogonia is apt to have relatively few receptive hyphae, while an infection with no spermogonia shows a rich development of these hyphae.

GREENHOUSE NOTES

Living infections have been studied in the greenhouse. The first macroscopic indication of the rust is the "fleck," a minute whitish spot on the leaf, less than a millimeter in diameter. Flecking begins six or seven days after inoculation. A day or two later both the upper and the lower surfaces of the infected area are yellow with the exudate, or nectar, from spermogonia, which is distinctly fragrant.

The infections grow rapidly. The size attained by an infection varies with the age and vigor of the leaf and with the number of infections on the leaf. Where infections are crowded together the individual infection remains small. The greatest size of the individual infection and the greatest deformation of host tissues occur on young tender leaves that carry one or only a few infections. Infected petioles of young leaves become hypertrophied, attaining as much as three times the normal diameter. On a young leaf blade the maximum diameter of an isolated infection is 8 or 10 mm. The infections often become irregular in outline, the spread being limited more or less by the larger veins. The leaf becomes distorted and thickened in the infected area and bulges upward. These hypertrophied tissues are under tension and, when slit for fixing, the strips spring apart.

Owing to the fact that relatively few host plants have been available for this work, it was necessary to fix material from time to time from the plants on which the greenhouse counts were made; therefore only a part of these infections have been followed through to old age. In the greenhouse records, infections are classified as singles (isolated infections), doubles (two in contact), and multiples (several confluent infections).

The greenhouse studies of the rust indicate that *Puccinia triticina* is heterothallic, for the isolated infection usually remains haploid and produces no aeciospores. An occasional single infection develops open aecia, perhaps fertilized by nectar carried by a thrip or a red spider or by chance contact with the nectar on another infected leaf beneath. Some of the infections situated fairly close together are singles at first, but during later growth become doubles or even multiples through confluence. These may develop belated aecia.

Nearly all the multiples and a large majority of the doubles produce open aecia between the eighteenth and the twenty-second day after inoculation. On a few of the inoculated plants the infections were studied and counted for a considerable period. On one plant with

11 single infections, all were sterile on the twenty-third day, although at that time 9 out of the 10 multiples on the same plant bore open aecia. On another host plant 19 out of 21 singles were sterile on the twenty-sixth day, at which time 4 out of the 5 doubles and the 4 multiples were producing aeciospores. On still another plant 19 out of 21 singles were sterile on the forty-second day after inoculation, at which time the 8 doubles and the 10 multiples were fertile. On yet another plant the 15 singles were still sterile on the forty-eighth day. The extreme case was a plant bearing 10 sterile singles 64 days old. At this time the infections and the leaves bearing them were dying.

The microscope shows that the majority of the isolated single infections bear both spermogonia and receptive hyphae in varying proportions, but the greenhouse studies show that the spermatia of a given rust plant can not fertilize the same plant; it is self-sterile.

More than the expected percentage of doubles produce aeciospores. In a heterothallic rust which is strictly bisexual, half of the infections would be (+) and half (−). In a random assortment half of the doubles would consist of a (+) and a (−), while the other half would consist of either two (+) or two (−). In other words, in 50 per cent of the doubles, (+) and (−) meet, and the combination should be fertile; while in the other 50 per cent, both members are of the same sex, and the combination should remain sterile. An actual count of doubles of ages ranging from 24 to 29 days gives 11 sterile and 31 fertile, or about 26 per cent sterile and 74 per cent fertile. A small amount of this is to be ascribed to accidental outside fertilization. In the data on single infections cited above, 4 out of 74, or about 5 per cent, bore aeciospores. Allowing a similar margin here for accidental fertilization, there is still a large surplus over the expected number of fertile doubles.

THE ISOLATED INFECTION

Material was available for a detailed study of the later history of the infection in which fertilization does not take place.

Reference has already been made (pl. 4, B, C, and D) to the emergent hyphae thrust between guard cells of stomata, and to other hyphae (pl. 6, A) that force a passageway between cells of the upper epidermis.

A hypha in a stomatal aperture is short-lived. Perhaps the exposure to the drier air outside of the leaf kills it. Perhaps, when the stoma attempts to close in the course of its daily stomatal movements, the hypha gets crushed. At any rate, the appearance of the inserted hypha soon changes. In Plate 5, A, the hypha (*b*), which grew down between the guard cells (*a* and *c*), takes the deep red stain characteristic of dead or dying protoplasm.

But this does not end the matter. Other hyphae have been massing in the air space above the stoma, and branches from these push down into the stoma alongside the first. Each stomatal hypha, in turn, dies and shrinks and another takes its place. In Plate 5, B, which represents a longitudinal section through a stoma of an 11-day sterile infection, five hyphae in succession have projected into and through the stomatal aperture. The two oldest (*b* and *e*) are dead and two others (*a* and *d*) are dying. Only the youngest (*c*), at the center of the group, is still fresh and vigorous. Plate 5, C, shows a

tangential (surface) view of such a stoma into which, as before, five hyphae in turn have grown. It is not uncommon to find one to six hyphae in almost every stoma in the infected area.

As stated earlier, the number of these stomatal hyphae in an infection varies more or less inversely as the number of spermogonia present. There are few in infections with numerous spermogonia and a great many in infections with no spermogonia.

There are apparent exceptions to this. In one infected area studied, which at first looked like one continuous infection, both spermogonia and emergent hyphae were abundant. But careful study of the upper epidermis showed the presence of four primary hyphae at different points (one of these is shown in pl. 2, E), so the apparently simple infection consisted really of four closely interwoven infections. It would be premature to say, however, that all such cases are of multisporidial origin. Only a long, careful study could determine that. As would be expected in the case cited above, fertilization had occurred at one or two points.

The amount of fungous growth back of a stoma at the time hyphae are pushing into the stomatal opening varies considerably. In Plate 5, B, the substomatal air space behind the stoma is but loosely threaded by scattered hyphae. In Plate 5, D (drawn at lower magnification than the preceding), where fresh hyphae are still being thrust into the stoma, the substomatal cavity (pl. 5, D, *b*) is filled with a dense fungous growth. It is noteworthy that the entire growth at this stage centers on the occupied stoma. Fertilization has not taken place; the cells are all uninucleate. It is at these points that aecia, fertile or sterile, arise.

In the absence of fertilization, this haploid substomatal growth continues for some time. Plate 5, E, shows a later stage in the development of the sterile aecium of an 11-day infection. The fungous mass has attained considerable size and has undergone the first differentiation into an upper half (*a*), of short, thick cells with dense cytoplasm and large nuclei, and a lower half (*b*), of large, loosely spaced cells with open vacuolate contents. In both parts the hyphae have become more or less disarticulated, each cell rounding up by itself. There are two stomata (*c* and *d*), both occupied by hyphae already dead. Due to the expansion of the cells and the disarticulation of the hyphae these emergent hyphae can no longer be traced into the body of the aecium. Fertilization did not take place; the aecium is still haploid.

Reference has already been made to the occasional formation of a thin layer of mycelial growth beneath the upper epidermis of the leaf and to the hyphae that push up from it between the epidermal cells. These are of much less frequent occurrence than are the hyphae within the stomata.

Under certain circumstances the development at the upper epidermis is much greater. In studying the living infections in the

EXPLANATORY LEGEND FOR PLATE 5

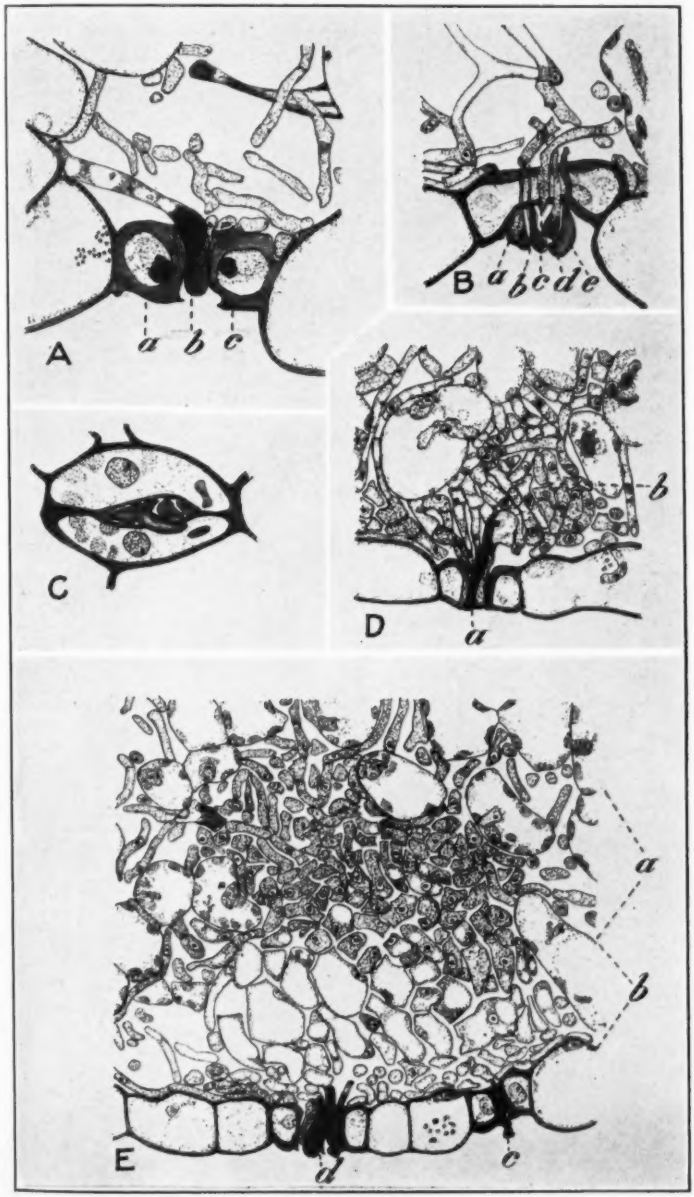
A.—Dying stomatal hypha (*b*) between the guard cells (*a* and *c*) in a 9-day infection. × 1,020.

B.—Longitudinal section of a stoma of an 11-day infection with 5 receptive hyphae, 2 dead (*b* and *e*), 2 dying (*a* and *d*), and 1 living (*c*). × 1,020.

C.—Surface (tangential) view of stoma from an 11-day infection. Within the stomatal aperture are 5 receptive hyphae, all dead. × 1,020.

D.—Nine-day infection. Stoma (*a*) filled with receptive hyphae, back of which is the young aecial primordium (*b*). × 640.

E.—Older sterile aecium from an 11-day infection, showing differentiation into a denser upper portion (*a*) and a more open lower half (*b*). Occupied stomata (*c* and *d*). × 640.



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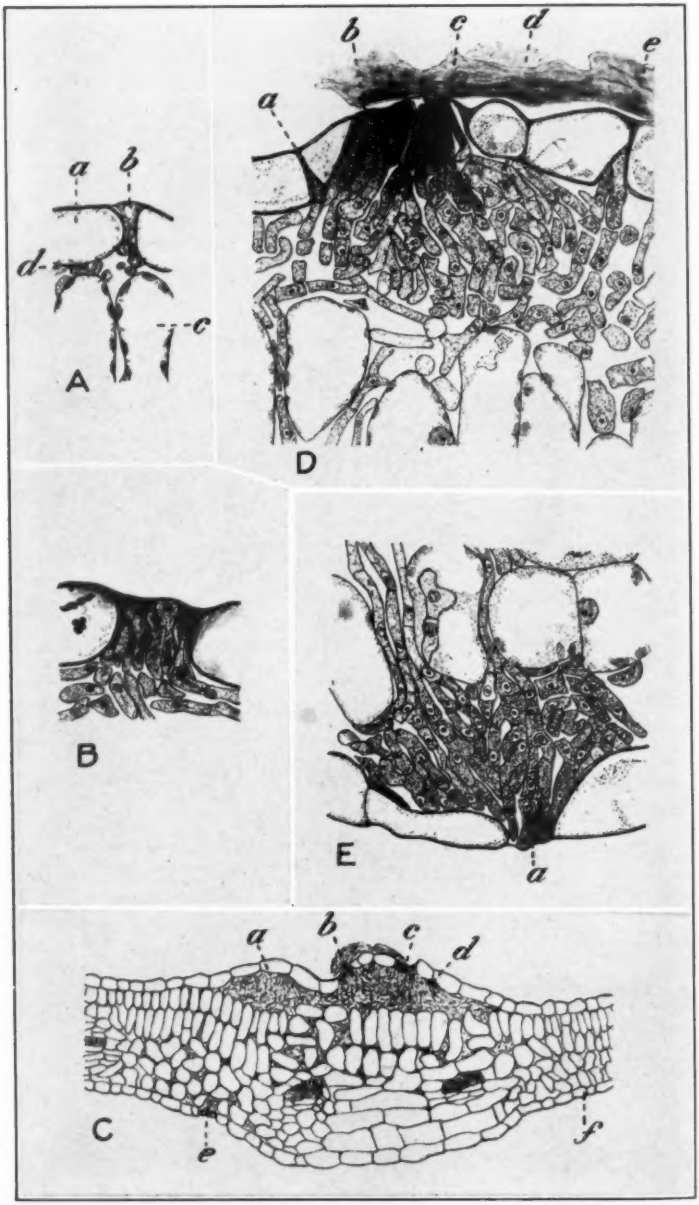

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greenhouse it was noted that infections on young, tender leaves make a more luxuriant growth and deform the leaves more than do infections on older, tougher leaves. Sections through the infections on young leaves show that a fairly massive fungous growth may form between the epidermis and the palisade layer. Plate 6, C, shows semidiagrammatically a section through a 9-day infection. There are no spermogonia in this infection. Under the upper epidermis is a continuous growth of hyphae, which at certain points (*b*, *c*, and *d*) has formed an upright palisade of parallel hyphae pushing between the epidermal cells. At *a* is a side section through a similar mass.

Plate 6, D, represents one of these (C, *b*) enlarged, showing the group of upright hyphae from *b* to *c* and later subsidiary attempts to pierce the epidermis at *a* and *e*. On the surface of the leaf is a dark-staining mass (*d*), presumably exuded either by the fungus or by crushed host cells.

In only one or two cases were similar but smaller subepidermal growths found at the lower surface of the leaf. One of these (pl. 6, E) occurred opposite a leaf vein where the absence of stomata prevented the formation of the ordinary receptive hyphae in stomata. Here, as before, several hyphae have squeezed in between epidermal cells, and others are growing in the same direction. The whole structure (pl. 6, E) is focused at *a*, where the hyphae have separated two epidermal cells. And here, as in the other cases, the cuticle of the outer epidermal wall is still intact, so that these hyphae are not really exposed to the outside air.

The nature of these anomalous structures is unknown, but since all gradations can be found, from the simple hypha between epidermal cells in Plate 6, A, to the more massive growth in Plate 6, D, it is probable that they are of the same nature. They occur in infections without spermogonia but with abundant hyphae in the stomata of the lower epidermis. The presumption is that the hyphae between epidermal cells, whether occurring singly or in groups, are receptive in nature. The similarity in structure between the group of hyphae in Plate 6, E, and the aecial primordium shown in Plate 5, D, is unmistakable.

The similarity stops at this point. No case has been noted of a sterile aecium developing either in connection with a single hypha between epidermal cells (pl. 6, A) or adjoining the more massive structures (D and E). In the absence of fertilization these structures, once formed, persist without developing further, so far as known, and become gradually decadent. Plate 6, B, shows a small group of these hyphae between epidermal cells from a 22-day infection. The cells are nearly dead and still haploid, and the mycelium beneath consists of a loose tangle of ordinary hyphae.

The early stages of growth of the sterile aecium (pl. 5, D) are centered on the receptive hyphae in a stoma. In the later development

EXPLANATORY LEGEND FOR PLATE 6

A.—Eleven-day infection. Between the upper epidermis (*a*) and the palisade layer (*c*) is a scant mycelial growth (*d*) from which a hypha (*b*) has grown up between epidermal cells. × 1,020.

B.—A group of similar cells, now dying, between epidermal cells in a 22-day infection. × 640.

C.—Diagram of leaf with 9-day infection showing subepidermal growth, bearing groups of upright hyphae between epidermal cells (*a*, *b*, *c*, and *d*). Receptive hyphae in stoma (*e*). Normal thickness of leaf (*f*). × 115.

D.—Group of upright hyphae (*b*–*c*) growing between epidermal cells of 9-day infection and subsidiary hyphae that attempted to grow between epidermal cells (*a* and *e*). Exudate (*d*). × 640.

E.—Similar group of hyphae emerging between cells of lower epidermis (*a*) next to a leaf vein in a 9-day infection. × 640.

the fungous growth above adjoining stomata may become confluent, forming one aecium. This is usually the case in the larger aecia. Plate 5, E, shows two occupied stomata (*c* and *d*). Plate 7, A, shows an aecium, from a 15-day sterile infection, with three sets of receptive hyphae in the stomata (*a*, *b*, and *c*) and others in adjoining sections.

The leaf tissues in this case have become hypertrophied. Plates 6, C, and 7, A, are drawn at the same magnification. The leaf now (pl. 7, A) is between two and three times the normal thickness as seen in Plate 6, C, *f*. The aecium (*d–e*) extends through more than half the thickness of the overgrown leaf.

The aecium is still haploid. All its cells are uninucleate (pl. 7, A), both the smaller ones in the upper region (*e*) and the large ones in the more open area below (*d*).

Plate 7, B, shows, at higher magnification, a strip through a large sterile aecium of a 30-day infection. It is still living, but decadent. Remnants of the stomatal hyphae may still be seen at *a*. The cells are uninucleate throughout.

In the great majority of the sterile aecia examined the cells remain uninucleate until death, but a few exceptions occur. In Plate 7, C, which shows a detail from a sterile aecium with every evidence of deterioration, there are a few multinucleate cells. Even these have an impoverished appearance, with scant cytoplasm and large vacuoles.

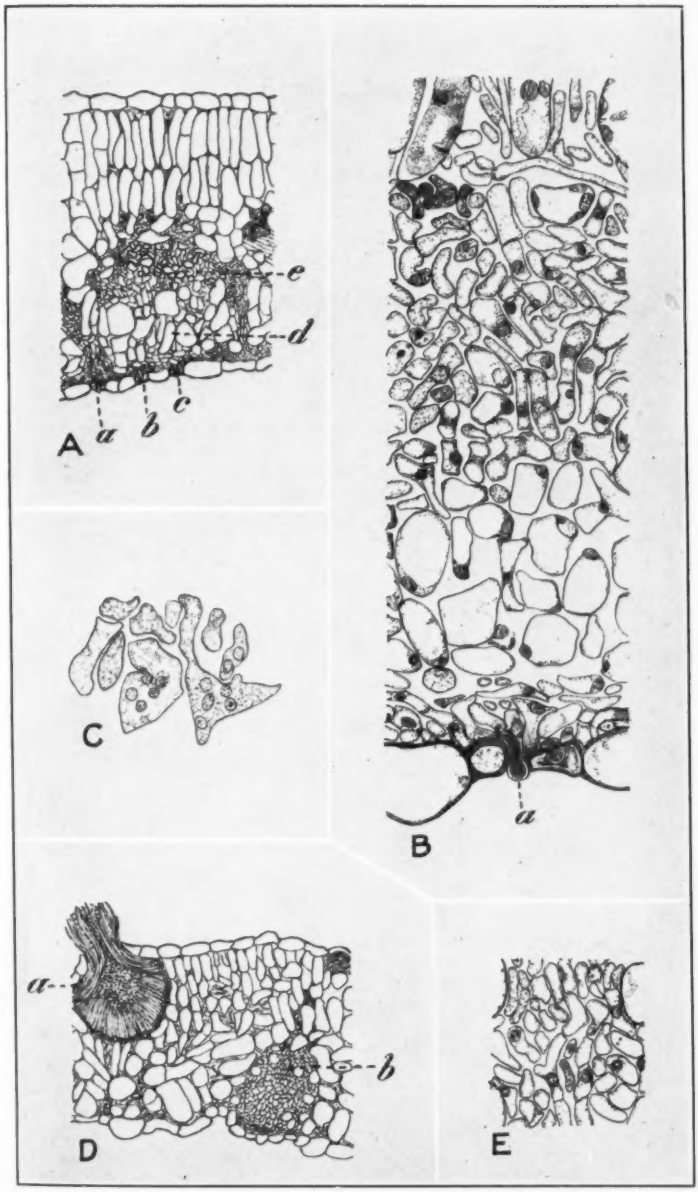
The amount of hypertrophy of the leaf and the size attained by the sterile aecium vary greatly in different infections. Plate 7, D, from a 22-day infection, shows a cross section of a leaf drawn at the same magnification as that shown in Plate 7, A. The contrast between the two in respect to the thickness of the leaves and the size of the aecia is marked. In one (pl. 7, A), an infection without spermogonia, the leaf is much thickened and the aecia are large; in the other (pl. 7, D), an infection bearing spermogonia, the leaf is less thickened and the sterile aecia are relatively small. The aecia here (D, *b*) are not only smaller but also less differentiated. A bit of the aecium shown in Plate 7, D, is enlarged in Plate 7, E.

In the end the aecia of the isolated infection die without producing spores. Plate 8, A, shows a small dead aecium of a 42-day infection. Even here the remnants of receptive hyphae can be seen at *a*.

It was noted earlier that infections of *Puccinia triticina* vary widely in the proportion of spermogonia to receptive hyphae. This difference persists into old age. One 42-day infection, a section of which is shown in diagram (pl. 8, B), bore 103 spermogonia, and only 23 small sterile aecia. Another 42-day infection of the same lot of material, shown in diagram (pl. 8, C), bore over 200 sterile aecia but had no spermogonia whatever. A comparison of the two diagrams shows other differences. They are drawn at the same magnification (× 33). In the spermogonial infection the hypertrophy of the leaf is slight; in the aecial infection it is much greater. The aecia of the spermogonial infection (pl. 8, B, *f* and *h*) are minute; those of the aecial infection

EXPLANATORY LEGEND FOR PLATE 7

A.—Semidiagrammatic drawing of cross section of a hypertrophied leaf bearing large sterile aecium, *d–e*. Stomata with emergent hyphae (*a*, *b*, and *c*); 15-day infection. × 115.
B.—Narrow strip through a large sterile aecium from 30-day infection. Receptive hyphae (*a*). × 640.
C.—Multinucleate cells from sterile aecium from 22-day infection. × 640.
D.—Diagram showing cross section of leaf with spermogonium (*a*) and sterile aecium (*b*). × 115.
E.—Enlarged detail from aecium in D. × 640.



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Less frequently, fertilization occurs at the upper epidermis. Plate 9, G, shows a hypha (*b*) that grew up between epidermal cells and pushed along for a short distance beneath the cuticle. Fertilization occurred, followed by sporophytic growth from the base of the cell. There are still 2 nuclei in the base of the hypha, and from 2 to 5 nuclei in near-by cells (*a*, *c*, and *d*).

Plate 9, H, shows an unusual case. Some minor injury to the leaf killed a single epidermal cell (*a*), and there are a few sporophytic cells (*b* and *c*) just beneath the dead cell. Apparently, fertilization was effected through the dead cell. It may be that the outside air, entering through the dead, withered host cell, served as the stimulus that attracted hyphae to this point. Perhaps it is a positive aerotropic response that brings all the emergent hyphae to the leaf surface, whether in stomata, between epidermal cells, or at dead epidermal cells.

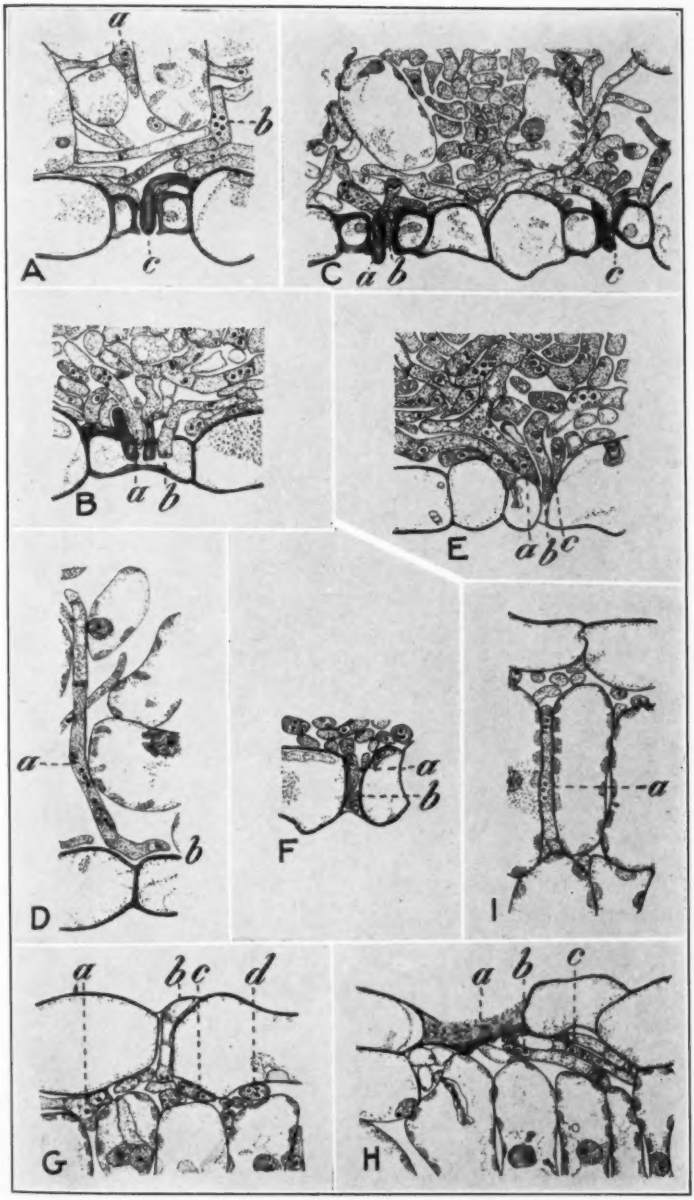
No aecial primordia have been found beneath the upper epidermis in these cases. The sporophytic hyphae grow down between the palisade cells and out into the spongy mesophyll. In Plate 9, G, the hypha at *a* is just pushing a branch into the space between two palisade cells. In Plate 9, I, the hypha at *a*, containing five nuclei, has almost reached the mesophyll. Other sporophytic hyphae have been found traversing the air spaces of the spongy tissue and above young aecia. No one sporophytic hypha can be traced from a receptive hypha at the upper surface to an aecium; but the probability is that the fertilizations at the upper leaf surface contribute to the sporophytic growth in the aecia beneath them, either by growth of new sporophytic hyphae or by progressive "diploidization" of the already existing mycelium, accomplished by successive nuclear divisions and migrations.

Soon after the introduction of spermatial nuclei into the receptive hyphae the haploid component of the aecial primordium becomes permeated throughout by scattered cells containing two or more nuclei. (Pl. 10, A.) The question arises as to the mode of distribution of the sporophytic generation from its point or points of origin.

Plate 10, B, shows a young aecium in which the haploid hyphae are still so loosely spaced that unusual opportunity is afforded for tracing out the course of individual hyphae. A group of hyphae centers on the stoma at *d*. One of these hyphae, which grew down from *a*, is now dead throughout most of its length, but near the stoma has given rise to a sturdy diploid hypha that has grown up to *e*, at which point it passes out of the plane of the section. Another diploid hypha growing upward from the receptive hyphae at the stoma is unbranched in its early progress but between *c* and *b* has given rise to five young

EXPLANATORY LEGEND FOR PLATE 9

A.—Stoma (*c*) with sporophytic cells (*a* and *b*) above it; 11-day infection. × 640.
B.—Longitudinal section of a stoma occupied by receptive hyphae, two of which (*a* and *b*) are multinucleate; 11-day infection. × 640.
C.—Fertilization at two stomata contributory to one aecium. Multinucleate receptive hyphae (*a*, *b*, and *c*); 11-day infection. × 640.
D.—Receptive hypha, *a*, with 11 nuclei, leading to stoma near *b* in next section; 11-day infection. × 640.
E.—Group of fertilized hyphae (*a*, *b*, and *c*) emerging between epidermal cells adjoining an aecium; 11-day infection. × 640.
F.—Fertilized emergent hyphae (*a* and *b*) between cells of lower epidermis. × 640.
G.—Emergent hypha (*b*) at upper epidermis with sporophytic cells (*a*, *c*, and *d*) near by; 11-day infection. × 640.
H.—Dead host cell (*a*) in upper epidermis with sporophytic cells (*b* and *c*) beneath. × 640.
I.—Sporophytic cell (*a*) growing down between palisade cells from subepidermal region; 11-day infection. × 640.



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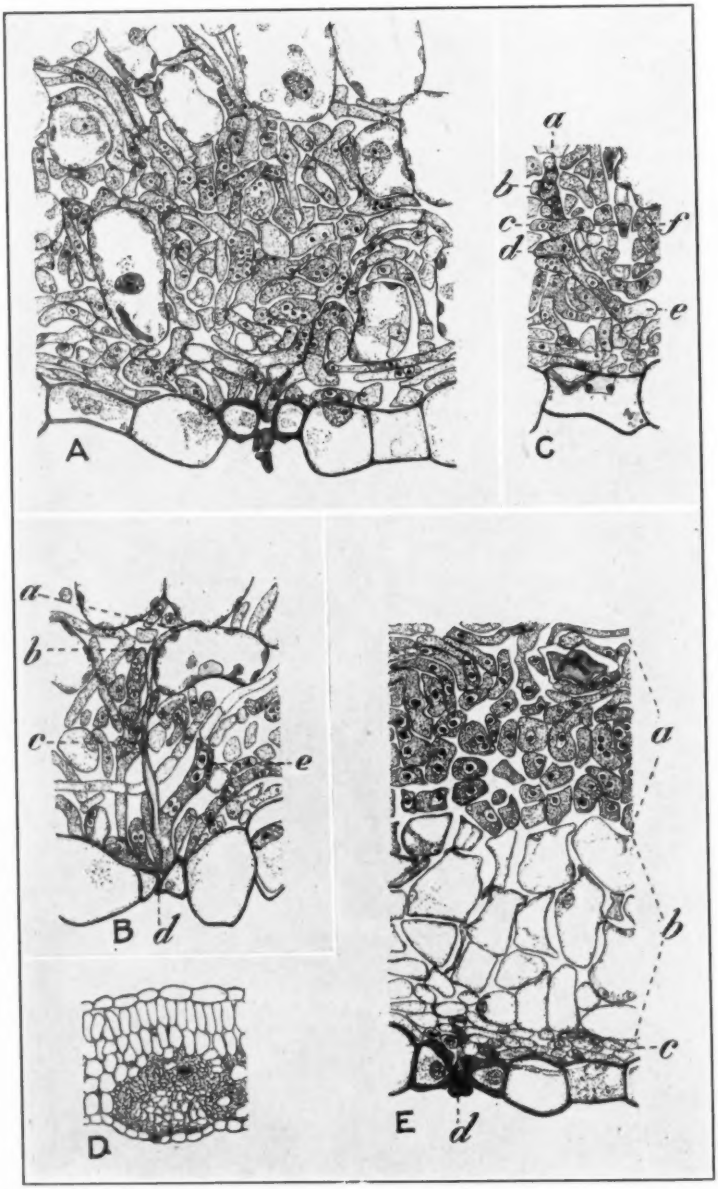

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branches. The acute angle that the branches make with the parent hypha shows that the direction of growth is away from the stoma, not downward toward it. Again, in Plate 10, C, from an older aecium with denser growth, a diploid hypha can be traced from *e*, not far from a stoma, upward, unbranched at first, then branching freely to right and left between *d* and *a*. It appears, then, that fresh growth from the fertilized stomatal hyphae distributes the new sporophyte generation through the upper area of the growing aecium.

In these cases (pl. 10, B, *a* and *c*, and C, *a*, *b*, *c*, *d*, *e*, and *f*) the cells are binucleate. Newly fertilized hyphae may contain from one to many introduced nuclei in addition to the single native nucleus. It is not clear how nuclear pairs could be established so quickly when many nuclei are introduced into a hypha. It may be that in cases like Plate 10, B, C, and E, where the sporophytic cells contain only 2 or 3 nuclei, comparatively few nuclei were introduced at the outset. And in cases like Plate 10, A, where cells with 2 to 9 nuclei are found dispersed throughout the aecium, and Plate 11, A, where nuclear conditions are still more extreme, the original addition of nuclei may have been more abundant.

In the aecium shown in Plate 10, A, there is a beginning of differentiation into areas; vacuoles are appearing in the cells of the lower part of the aecium. This change affects gametophytic and sporophytic cells alike throughout this area.

Plate 10, D, represents semidiagrammatically a somewhat older aecium in which this differentiation is well established. There is now an area in the lower part of the aecium, shaped like a thick biconvex lens, composed of large, nearly empty cells that are rapidly dying. This area is capped by a thick layer of smaller, denser cells that will later give rise to the spores. A median strip through this aecium from top to bottom is shown enlarged in Plate 10, E. The contrast in cell size and cell content between the sporogenous area (*a*) and the "space-making" area (*b*) is marked. Remnants of hyphae are still present in the stoma (*d*), but it is no longer possible to trace any connection between them and the upper part of the aecium.

The sporophytic cells (pl. 11, A) of the sporogenous area (*a*–*c*) contain from 2 to 12 nuclei. There are also multinucleate cells in the space-making area (A, *b*), but they are dying. It seems probable that the abundance of nuclei in this case (A) has been continuous since fertilization, especially as younger aecia can be found with the same nuclear condition; but this can not be taken for granted. It is possible that at an intermediate stage rapid growth and cell division reduced the number of nuclei per cell and that later the growth conditions in the sporogenous area favored rapid nuclear divisions, giving rise to the condition found here.

From one cause or another, the average number of nuclei in sporophytic cells of aecia of this age varies widely. Sooner or later, how-

EXPLANATORY LEGEND FOR PLATE 10

A.—Young fertile aecium showing mixture of gametophytic and sporophytic cells. Sporophytic cells with two to nine nuclei; 11-day infection. × 640.

B.—Young fertile aecium showing growth of sporophytic hyphae (*e* and *b*–*c*) from fertilized hyphae at stoma *d*; dead hypha at *a*; 11-day infection. × 640.

C.—Portion of young fertile aecium with sporophytic hypha growing from *e*, near stoma, to *a*, with branches (*b*, *c*, *d*, and *f*); 11-day infection. × 640.

D.—Diagram of older fertile infection from 11-day infection. × 115.

E.—Portion of aecium from D enlarged, showing sporogenous area (*a*), the space-making area (*b*), finer hyphae (*c*), and remnants of receptive hyphae (*d*). × 640.

ever, the binucleate condition prevails in the sporophytic component of the aecium. This may come while the aecium is still young or may be deferred until spore formation has begun. In the newly fertilized emergent hypha, 1 native nucleus and from 1 to 10 introduced nuclei have been observed. In the final binucleate condition, a nuclear pair is supposed to consist of one male and one female nucleus. Just how the original disparity is changed into the final equality and regularity is not clear. It could be achieved by an unequal rate of nuclear division in the native and introduced nuclei. It may be accomplished by supplementary fusions with haploid cells, although no evidence of this has been seen.

Soon after the stage represented in Plate 11, A, cells of the sporogenous area begin to grow downward into the space-making area to form the initial cells of the spore chains. Plate 11, B, represents an aecium from an 11-day infection in which the layer of basal cells (*a*) is just forming.

Details from the layer of basal cells of this aecium and others of the same stage are shown in Plate 11, C to H. When a cell in the sporogenous area pushes down to assume the function of a basal cell, it may grow from one end or from any point along its length. Plate 11, G, shows several young basal cells, some binucleate and some multinucleate. In three of these the basal cell is merely a continuation of the ordinary apical growth of the hypha. In the fourth (pl. 11, G) the hypha is pushing down at two points (*a* and *b*). Plate 11, E, represents another group of young basal cells. The penultimate cell of one (*a*) is pushing out a branch to form a second basal cell. In Plate 11, H, in which a young spore chain is initiated, one of the upper cells of the hypha (*a*) is pushing out a branch. This multiplication of basal cells by branching is common. In Plate 11, J, from an older aecium, the common stem (*a*) has given rise to two main chains of spores, with a younger one starting at *c* and a possible fourth pushing out at *b*.

When a cell extending horizontally pushes down a branch from its center to form a basal cell, the result simulates the 2-legged cell described in rust literature. In Plate 11, D, the irregular cell (*a–b*) has started to form a branch at *c*. In Plate 11, C, the branch (*c*) is somewhat longer. In Plate 11, F, the centrally placed branch (*a*) of the curved cell (*c–b*) has become a fully formed basal cell with one spore mother cell. Figures like this have been interpreted as meaning that two haploid cells (as in pl. 11, F, *c* and *b*) fused at their tips, then grew down from the point of fusion to start the diploid spore chain.

EXPLANATORY LEGEND FOR PLATE 11

A.—Portion of sporogenous area of aecium from 16-day infection. Multinucleate cells (*a*, *b*, and *c*) × 640.

B.—Diagram of aecium with basal cells forming at *a*; 16-day infection. × 115.

C.—Early stage in formation of basal cell (*c*) from center of curved cell (*a–b*). × 640.

D.—Irregular cell (*a–b*) from sporogenous area. Beginning of basal cell (*c*). Sixteen-day infection. × 640.

E.—Detail from sporogenous layer showing young multinucleate basal cells. Branch forming at *a*. Sixteen-day infection. × 640.

F.—Two-legged basal cell (*a*, *b*, and *c*); 16-day infection. × 640.

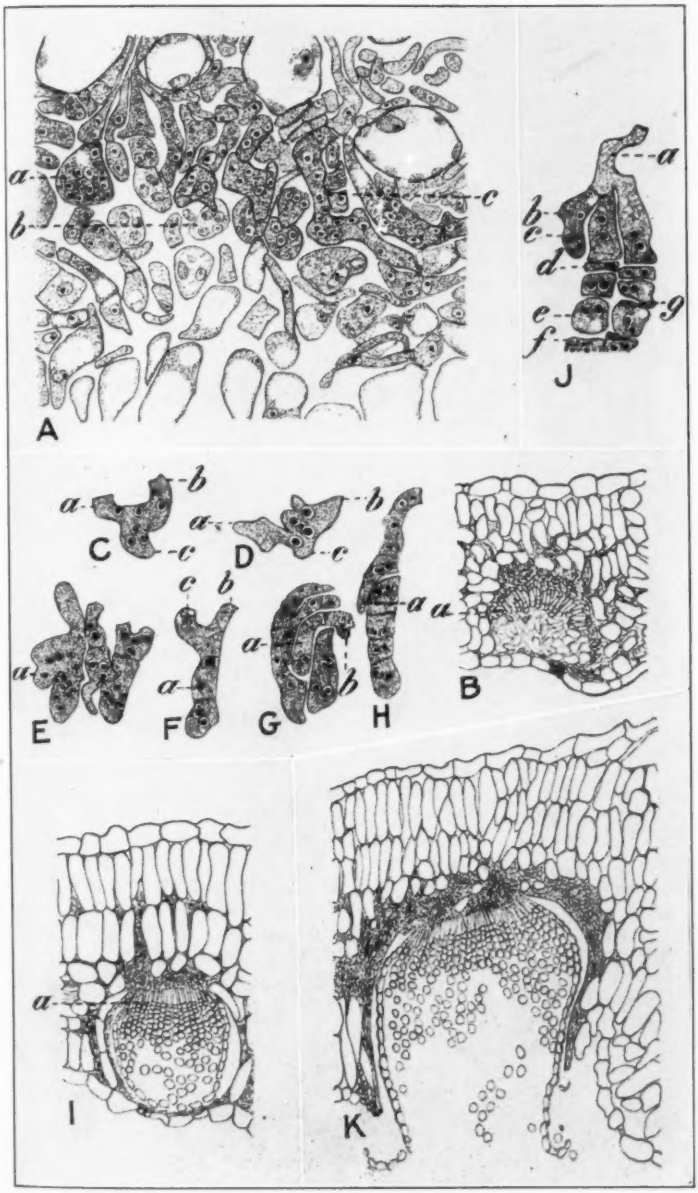
G.—Group of young basal cells containing two to six nuclei. Hypha (*a–b*) pushing down at two points. Sixteen-day infection. × 640.

H.—Young spore chain branched at *a*; 16-day infection. × 640.

I.—Diagram of maturing aecium with basal cells at *a*; 16-day infection. × 115.

J.—Detail from aecium in I showing two spore chains from one hypha (*a*); a third spore chain started at *c*, and perhaps a fourth at *b*. Spore mother cells (*d* and *e*) and intercalary cells (*f* and *g*). Sixteen-day infection. × 640.

K.—Diagram of open aecium from 20-day infection. × 115.



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Such an interpretation would be obviously incorrect in *Puccinia triticina*, as the sporophyte generation started long before these cells were formed.

When first formed, the basal cell may contain from two to eight nuclei. (Pl. 11, E and G.) An even number of nuclei is usually found, but these are scattered irregularly, not in pairs. The extra nuclei are utilized in supplying nuclei to the first cells of the spore chain, and by the time the chains are well established the basal cells are regularly binucleate. (Pl. 11, J.)

The first cells formed by the basal cells are the spore mother cells. Each of these divides into two—a large cell, the definitive spore, and a very small intercalary cell. The intercalary cell (pl. 11, J, *f* and *g*) is seen attached to the spore as an inconspicuous slice from one side.

As the parallel, closely packed chains of spores from the basal cells lengthen, they push down into the area of looser dead cells below, crushing them as they grow. The peripheral layer of basal cells gives rise to the peridium, a continuous sheath, one cell thick, inclosing the spore mass. Plate 11, I, shows a maturing aecium of a 16-day infection, and Plate 11, K, an open aecium from a 20-day infection. The diagrams in Plate 11, B, I, and K, are drawn at the same magnification and show the great increase in the size of the aecium after spore formation sets in, and also the increase in the thickness of the host leaf bearing it.

DISCUSSION

The entry of the sporidium into the epidermal cell and the formation of the primary hypha and its mode of branching are very similar in *Puccinia graminis* (*1*) and *P. triticina*.

Older primary hyphae and older haustoria of *Puccinia triticina* become incased in a layer of material staining like the host cell walls and presumably deposited by the host cell. In the case of some invading parasites, e. g., *Ophiobolus graminis* Sacc. on wheat roots (*11*), wall materials are continually built up around the advancing tip of a hypha as it forces its way into a cell, resulting in the finger-shaped "lignituber," through the end of which the hypha may finally escape into the cell lumen. In *P. triticina* entrance into a host cell appears to be unimpeded, but later the fungus may become insheathed by wall materials. This sheath is probably a partial defense of the host against the intruder.

Dodge (*10, p. 1751*) and others "choose to view today the picture of the rust life cycles against the background of a red alga ancestry." The finding of functional receptive hyphae in the rusts adds interest to such comparisons and increases the probability of phylogenetic relationship between the two groups. No generalizations can be made, however, until it is known how widespread and how varied this mode of fertilization is among the rusts.

In *Puccinia triticina* functional receptive hyphae form at both the upper and the lower surface of the leaf. By far the greater number, however, are in the stomata of the lower surface, and it is here that fertilization ordinarily takes place. Fertilization is doubtless aided by the distribution of the spermogonia, at least half of which open upon the lower surface of the leaf. Because of this, visiting insects,

attracted by the nectar and going from one infection to another, may transfer spermatia to the lower as well as to the upper surface of the leaf.

Aecia, so far as noted, form only in connection with stomatal hyphae at the lower surface of the leaf. No sterile aecia have been found beneath the upper epidermis. Rarely a fertile aecium opens on the upper surface, but since the history of its development is unknown it is uncertain whether it started with a fertilization through the upper epidermis or through stomata of the lower epidermis. Fertilization takes place occasionally at the upper epidermis, but sporophytic hyphae initiated there apparently become effective only by growing downward and becoming part of an aecium below.

The fertilization of emerging hyphae at points some distance from an aecium and the spread of sporophytic mycelium from these points may explain some of the hitherto baffling cases of intermingled sporophytic and gametophytic mycelia in the rusts. Sporophytic hyphae have been found mingled with the mycelium of the gametophyte generation by Blackman and Fraser (*4*), Olive (*17*), Lindfors (*15*), Walker (*19*), Allen (*1*, *2*), and others.

The word "spermogonium" implies a male structure. It should not be forgotten, however, that when nectar is interchanged between two infections of different sexes each fertilizes the other. Genetically, as Craigie (*8*) pointed out, the spermogonia of one infection are of one sexual group, while the spermogonia of the other infection are of another group.

The majority of the infections bear both spermogonia and emergent hyphae, so the haploid generation must possess the genetic basis for both structures. The proportion of spermogonia to emergent hyphae, however, varies widely in different cases, some infections bearing many spermogonia and few receptive hyphae, others fewer spermogonia and more receptive hyphae, and a few no spermogonia and many receptive hyphae. Whether this is due to differences in the environment or to genetic differences in the individuals is not certain. The host plants were given similar treatment. Infections of the same age, borne on the same host plant at the same time and resulting from the same inoculation, showed the full range of variation. As great a variation in the proportion of spermogonia to aecia was found in infections on younger leaves as was found on older leaves. If this variation is the result of varying conditions in the host or in the external environment, it is not clear what factors could be responsible for it.

On the other hand, data at present available are not sufficient to prove that the differences are genetic in origin. Of interest in this connection is the fact that an isolated gametophyte, although possessing both male and female organs, remains haploid throughout its life, and that three-fourths of the double infections (instead of the expected one-half) prove fertile. The number of doubles under observation was not great enough to give much weight to this evidence, but it at least suggests that in *Puccinia triticina* more combinations prove fertile than would be expected if the infections could be divided exactly into two equal groups, one (+) and one (−). Further experiments directed toward a genetic analysis of the situation might reveal varying degrees of genetic "maleness" and "femaleness" expressed morphologically in the varying proportions of male and female organs. This might determine the range of mating capa-

bilities and so affect the percentage of fertile doubles. Hartmann (*13*) and Kniep (*14*) have summed up a number of more or less similar cases found among the algae and fungi.

In hybridizing varieties of *Puccinia graminis*, Stakman, Levine, and Cotter (*18*) obtained several physiologic forms from the spores of one aecial cup. On the other hand, in crossing physiologic forms, Newton, Johnson, and Brown (*16*), as a rule, isolated but one physiologic form from one aecium. The occurrence of only one or of more than one physiologic form depends on whether the sporophytic growth in any one aecium is descended from a single pair of conjugate nuclei (or several similar pairs) or from several unlike pairs. In *P. triticina* the mechanism of fertilization is certainly adequate to permit several physiologic forms to enter into the composition of one aecium. With several hyphae emerging at one stoma and several occupied stomata underlying one aecium, and with occasional accessory points of fertilization between epidermal cells of the lower epidermis and a still further possible contribution of fertile hyphae from a fertilization at the upper epidermis, there is ample opportunity for the introduction of spermatial nuclei. Any one of these receptive hyphae may receive from one to a dozen outside nuclei. But while the facilities for fertilization would permit the introduction of several physiologic forms, it would be uncommon in nature for spermatia of several forms to mingle on so small a part of the leaf surface as to enter the same aecium.

The mechanism for the distribution throughout the aecium of the introduced nuclei is not fully worked out. Buller (*5*) finds that in *Coprinus lagopus* Fr., when two haploid mycelia of opposite sex meet, there is a progressive diploidization of each mycelium by the other, effected by repeated nuclear divisions and migrations. A hypha of one mycelium touches a hypha of the other mycelium, fuses with it, its nucleus moves over, then divides, and one of the daughter nuclei moves on into the next cell. This in turn divides, sending on one of the daughter nuclei into the next cell. In this way both mycelia are rapidly transformed into diploid mycelia, each cell of which has a pair of conjugate nuclei of opposite sex.

In *Puccinia triticina* an indefinite number of nuclei may be introduced into the haploid receptive hypha. Branches from this hypha originate either near the stoma or farther up in the aecium. These branches may be binucleate from the beginning, but are often composed of multinucleate cells, the nuclei of which look alike. After the initial difference in size between spermatial nuclei and aecial nuclei is lost, there is no means of identifying male and female nuclei. The nuclei do not lie in pairs, but are scattered irregularly through the cytoplasm. Eventually, the binucleate condition is achieved by a more rapid rate of division of the native nuclei, by nuclear migrations, or by cell fusions between adjoining cells. These processes have not been seen, but their existence seems probable. From what is known of other fungi with conjugate nuclei it may be concluded that in the aeciospore one nucleus is of male and the other of female descent, but it would not be possible to prove this in *P. triticina*.

In *Puccinia triticina*, sporophytic growth in the aecium is well established before the layer of basal cells is differentiated. As a consequence, basal cells are formed, ordinarily at least, by cell

division and not by fusion. Sometimes a false appearance of fusion is given when a long horizontal cell pushes down a branch at its center to form the basal cell, thus simulating the 2-legged cell described in rust literature. For many years 2-legged cells have been interpreted as meaning that two haploid hyphae meet at their growing tips, fuse at the point of contact, and give rise at this point to a downward-growing binucleate cell, but it is evident that the presence of a 2-legged basal cell is not in itself proof of fusion. In the past the presence of a few 2-legged cells in the layer of basal cells has sometimes been the only evidence offered that the sporophyte originated by fusion of pairs of uninucleate cells in the sporogenous area in the aecium. Without a study of younger stages, there is a risk of error in this assumption.

SUMMARY

Puccinia triticina Eriks., the leaf rust of wheat, has its gametophyte generation on species of Thalictrum. The sporidium, formed by the germinating teliospore in the spring, is a haploid spore, and when it falls upon a Thalictrum leaf it germinates, enters directly through the outer epidermal wall, and forms a 4-cell to 6-cell primary hypha in the epidermal cell, which in turn gives rise to haploid intercellular mycelium.

After six or seven days of vegetative growth, reproductive activities set in. Spermogonia form in about equal numbers at the two surfaces of the leaf. At the same time receptive hyphae grow into stomatal apertures or between epidermal cells of the upper or the lower epidermis. On young tender leaves an extensive growth of mycelium may be present beneath the upper epidermis, giving rise to groups of upright hyphae pushing up between epidermal cells.

Puccinia triticina is heterothallic. A monosporidial infection may bear both spermogonia and receptive hyphae, but if kept isolated it remains haploid. Three-fourths of the double infections (i. e., two in contact) produce open aecia.

A comparative study of infections of the same age shows that some have many spermogonia and few receptive hyphae, others have comparatively few spermogonia and more receptive hyphae, and a few have no spermogonia and abundant receptive hyphae.

In the substomatal air space above a stoma occupied by one or more hyphae, other hyphae grow and branch rapidly, forming a dense little nest of cells, the beginning of an aecium. In the absence of fertilization this haploid aecium grows, undergoes the first differentiation into areas, then slowly deteriorates, and dies without forming spores.

When spermatia from another and different infection are brought to an infected area, spermatial nuclei enter the tips of the receptive hyphae. There may be from 1 to 10 spermatial nuclei in each of these hyphae. Fertilization takes place most frequently at the stomata, but may occur also in hyphae emerging between epidermal cells of the lower and the upper surface of the leaf.

Growth from these fertilized cells permeates the aecium. Cells of sporophytic hyphae may contain 2 nuclei, but usually contain more—sometimes 8 or 10. This multinucleate condition may persist

during the organization of the sporogenous area. Young basal cells contain from two to eight nuclei. The extra nuclei are utilized in the formation of binucleate spores, and eventually the basal cells become regularly binucleate.

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RESTORATION OF VIRULENCE OF ATTENUATED CURLY-TOP VIRUS BY PASSAGE THROUGH STELLARIA MEDIA[1]

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INTRODUCTION

In preliminary reports the writer (*6*, *8*)[3] announced briefly the restoration of the virulence of sugar-beet curly-top virus after it had been attenuated by a very resistant host. What appear to be similar cases of this phenomenon with pathogenic organisms that have become attenuated have been reported frequently in animal pathology. For example, Pasteur, Chamberland, and Roux (*9*) reported that exposing the anthrax organism to a certain degree of heat so reduced its infective power that it was barely able to kill day-old guinea pigs. However, when the attenuated organism was passed successively through several day-old guinea pigs, it gradually increased in virulence until finally it was able to cause the death of a sheep.

The term "attenuation," as used in this paper with reference to curly-top virus, means the reduction in the ability to infect and in the power to produce severe symptoms. Of the two criteria used in determining attenuation, the mildness of symptoms produced is considered more important than the percentage of infection obtained.

In plant-disease literature there are several examples that appear to be cases of virus attenuation. Johnson (*4*), by means of heat, succeeded in attenuating the mosaic virus of tobacco to such an extent that it produced only a small percentage of infection and very mild symptoms as contrasted with the effect produced by the untreated virus. He was unable to restore the virulence to this virus by repeated passages through susceptible tobacco plants. Later Johnson and Ogden (*5*) succeeded in occasionally producing an attenuated condition of tobacco mosaic virus by bubbling air and oxygen through the extract of green mosaic plants. This attenuation of the virus was similar to that produced by heat, remaining stable through several subsequent transfers. These two cases of virus attenuation correspond closely to the kind of attenuation discussed in this paper.

Salaman (*10*) reports modification of the symptoms of crinkle A, a virus disease of potato, after the virus had passed through Datura. These symptoms did not resemble a mild form of the original crinkle A, but rather a mild mosaic. When transferring crinkle A to several varieties of potatoes by grafting he secured crinkle on some varieties but mosaic symptoms or acute streak symptoms on others. While the evidence is inadequate to settle the problem definitely, he suggests the possibility that he was working with a mixture of viruses.

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[2] Grateful acknowledgment is made to Eubanks Carsner and N. J. Giddings, senior pathologists, Division of Sugar Plant Investigations, for the many suggestions given during the progress of this work and for their helpful criticisms of the manuscript.

[3] Reference is made by number (italic) to Literature Cited, p. 765.

Smith (*12*), working with the crinkle virus of potatoes, passed it through *Datura stramonium* L. and then inoculated potato plants. Instead of modified crinkle symptoms, mild mosaic mottling resulted. In his earlier work on potato mosaic, Smith (*11*) concluded that the various symptoms appearing on different plants inoculated with the mosaic were due simply to variation in host response. In his later work (*12*) on crinkle of potatoes he suggested the presence of more than one virus.

Carsner (*1, 2*) reported the first cases of attenuation of the curly-top virus by resistant hosts, among which was *Chenopodium murale* L., in 1919 and 1925. After this Carsner and Lackey (*3*) reported the attenuation of the curly-top virus when it was passed through very resistant sugar beets. Repeated transfers through susceptible sugar beets failed to restore the virulence to the attenuated virus. Later Lackey (*6, 7*) recorded additional evidence of this attenuation reaction of curly-top virus when passed through certain resistant host plants.

The idea was conceived that if a resistant host attenuates the virus an exceedingly susceptible host might restore its virulence. Carsner (*1*) had reported chickweed, *Stellaria media* (L.) Cyr., as a very susceptible host plant, so it was selected for trial.

It has been recorded in literature that the virulence of some plant viruses has been increased by methods of handling. Smith (*11*) reported that potato mosaic increased in virulence when it was transmitted to healthy White Burley tobacco by the aphis *Myzus persicae* Sulz. He also found that tobacco ring spot was increased in virulence by progressive inoculation through successive generations of susceptible tobacco. This increase continued only up to a certain point and then the virus showed a tendency to revert to its original virulence. Later Smith (*12*) found that the crinkle virus of potatoes could be passed through successive generations of tobacco and a severe leaf-drop streak obtained which readily killed some varieties of potatoes. The interveinal mosaic of potatoes when passed through tobacco also came out as a severe leaf-drop streak. Smith (*12*) suggests that probably more than one virus is involved, and states: "It may be suggested then that passage of tobacco merely liberates in some way the streak virus which was already present, so that it attacks every potato variety inoculated." It is difficult to determine whether one virus was increased in virulence or whether the different hosts suppress one virus and liberate another.

DOSAGE EXPERIMENTS

It has been suggested that the reduction in percentage of infection and possibly the variation in type of symptoms produced by the apparently attenuated curly-top virus might be attributed to mere reduction in the quantity of virus involved. The results of the following experiment bear on this problem.

Attenuated virus was obtained by inoculating nettle-leafed goosefoot, *Chenopodium murale*, with 50 to 75 leaf hoppers, *Eutettix tenellus* (Baker), carrying the virulent form of virus (*2*). After five to six weeks these hoppers were removed. The virus was transferred from the Chenopodium to test beets, *Beta vulgaris* L., either by placing nonviruliferous adults on the plant or by using the nymphs that hatched

out on the Chenopodium. The nymphs gave the same results as the adults.

The virulent virus, for comparison, was transmitted to test beets by means of leaf hoppers reared on a severely diseased beet.

The beets used in this experiment to test the virulent and attenuated virus were susceptible plants in the fourth true leaf stage, and the leaf hoppers from the two sources, beet and Chenopodium, were caged on the young beets for three days. These insects were confined on a true leaf of each beet by means of celluloid cylinder cages. Two leaf hoppers to a plant were used in some cases and 10 leaf hoppers in others. The results obtained are given in Table 1.

TABLE 1.—*Results of inoculation of susceptible sugar beets with different numbers of leaf hoppers carrying either the attenuated or the virulent form of curly-top virus*

[Leaf hoppers caged on beets for four days]

Number of insects used and form of virus	Beets inoculated	Beets infected		Type of symptoms
	Number	*Number*	*Per cent*	
2 leaf hoppers with attenuated virus	22	8	36	Mild.
10 leaf hoppers with attenuated virus	38	21	55	Do.
2 leaf hoppers with virulent virus	41	29	71	Severe.
10 leaf hoppers with virulent virus	29	24	83	Do.

This experiment, confirming previous work with *Chenopodium murale*, shows the attenuated nature of the virus obtained from this host plant. It shows also that the symptoms were mild irrespective of whether 2 or 10 leaf hoppers were used. It is to be noted that 10 leaf hoppers bearing the attenuated virus from Chenopodium produced 55 per cent infection, while 2 leaf hoppers harboring the virulent virus produced 71 per cent infection, severe symptoms being produced in all cases where the virulent virus was concerned.

In carrying along a stock of the attenuated virus, from 50 to 80 leaf hoppers have been caged on young beets in the fourth to sixth leaf stage and the resulting symptoms have remained mild; on the other hand, 1 leaf hopper harboring virulent virus has produced severe symptoms on a young growing beet if it succeeded in infecting the beet at all.

It is evident from these results that the greater the number of insects used to inoculate the beet the greater is the percentage of infection obtained. It is also apparent that 2 leaf hoppers carrying virulent virus may cause a higher percentage of infection and severer symptoms than 10 insects carrying attenuated virus. Since as great a number as 80 leaf hoppers with the attenuated virus fail to produce severe symptoms on young growing beets, while 1 hopper carrying virulent virus will produce severe symptoms, it is concluded that in experiments of this type the dosage of virus is not a factor controlling the subsequent symptoms produced.

RESTORATION STUDIES

The plants used in the experiments in the restoration of virulence were *Chenopodium murale*, *Stellaria media*, and the susceptible sugar beets used as test plants and as checks.

Figure 1, which is a diagram giving data from a typical experiment, illustrates the general plan used in attenuating the virus and restoring its virulence, the original virus being in each case the virulent form of stock virus maintained on susceptible sugar beets.

A susceptible beet was used in the beginning to maintain a stock culture of virulent virus. Fifty leaf hoppers from this culture were used to inoculate a *Chenopodium murale* plant.[4] After a period of six weeks, nonviruliferous leaf hoppers were fed on this plant for a week. Four of these hoppers bearing the attenuated virus from the *Chenopodium murale* were used to inoculate a chickweed, *Stellaria media*.[5] This chickweed was a young, fast-growing plant with three stems. The four insects were caged on the plant by means of a small, cylindrical, celluloid cage. The cage was left on for three days. During the incubation period of the disease the chickweed was kept in a semishaded part of the greenhouse under moist and good growing conditions. Six days after inoculation curly-top symptoms became visible. Nonviruliferous leaf hoppers were then caged on the diseased chickweed for three days. These leaf hoppers were then transferred to young healthy beets which were in the 2-leaf and 4-leaf stages, 2 leaf hoppers being used to a plant. In this manner 37 beets were inoculated, 29 of which became infected and showed severe symptoms. At the same time 7 young beets were inoculated with leaf hoppers from the beet on which the stock culture was maintained and 33 beets were inoculated in like manner with virus from the Chenopodium plant. Six of the 7 beets (fig. 1) inoculated with the virus directly from the beet (virulent) became infected and showed severe symptoms. These symptoms were indistinguishable from those produced by the virus obtained from the chickweed. On the other hand, of the 33 beets inoculated with virus directly from Chenopodium, only 6 became infected and these showed very mild symptoms. The tests with the virus from Chenopodium show that the virus used to inoculate the chickweed was attenuated. From the fact that 29 of the 37 test-beet plants infected with this attenuated virus after it has been passed through chickweed had

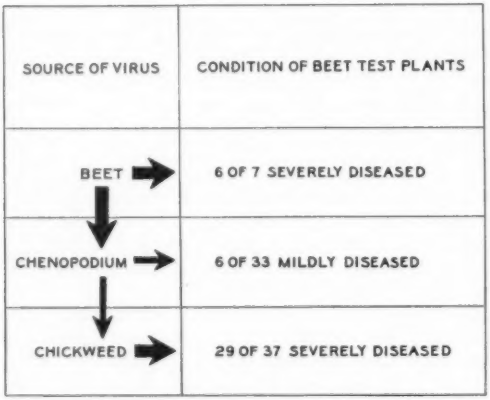

FIGURE 1.—Plan used in the attenuation and restoration of the curly-top virus, with data from a typical experiment. The arrows indicate the successive passage of the virus through the different host plants, the heavy arrows representing the virulent form of the virus and the light arrows the attenuated condition

[4] Plants used in the course of this work were approximately 6 weeks old and 4 to 5 inches high. As Carsner (2) has shown, in some cases no virus can be secured from such inoculated plants, while in other cases only attenuated virus is obtained.

[5] Plants used were approximately 6 weeks old with stems ranging from 1½ to 2 inches in length. It should be noted that passing the attenuated virus through chickweed does not always result in complete restoration of virulence. The critical factors influencing restoration have not been determined.

symptoms indistinguishable in severity from those produced by the original virulent virus, it is evident that the virus, after being attenuated, had been restored to its original virulence.

The symptoms produced by the virus from the three sources are shown in Figure 2.

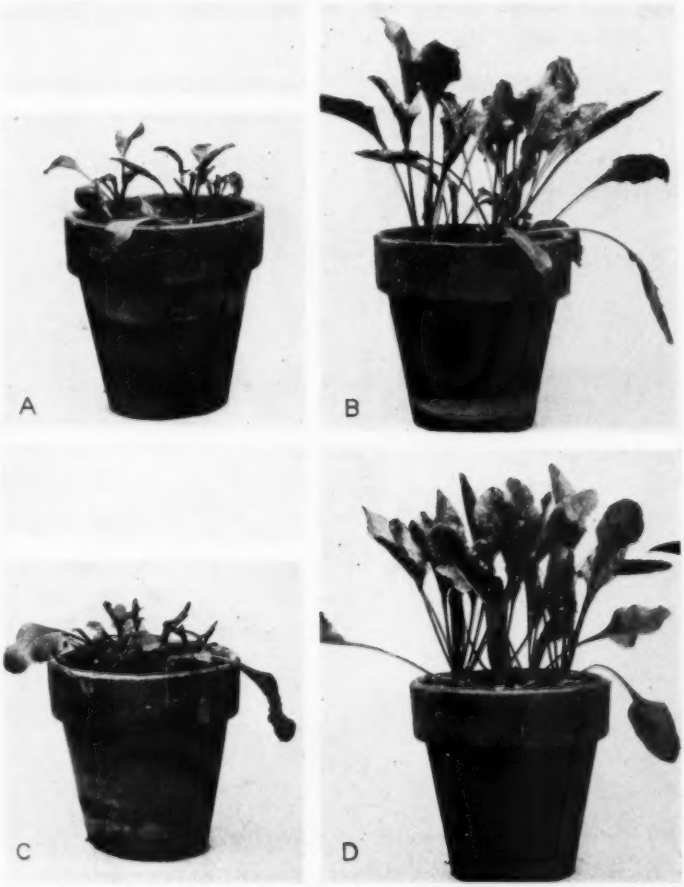

FIGURE 2.—A, Beets inoculated with the original virus; B, beets inoculated with the same virus after attenuation by *Chenopodium murale;* C, beets inoculated with this virus restored to its original virulence by passage through *Stellaria media:* D, normal uninoculated plants

The test beets used were grown under similar conditions from a seed stock of known high susceptibility, and were all of the same age. The illustration shows the beets seven weeks after inoculation. The beets in pot A were inoculated with the original virulent virus. Those in pot B show the effect of this virus after its attenuation by passage through *Chenopodium murale.* The disease is mild, the

plants showing only slight vein distortion and curling of the younger inner leaves and no shortening of petioles or dwarfing. In contrast, the plants in pot C show the effect of this virus after its subsequent passage through chickweed. The symptoms, extreme dwarfing accompanied by severe curling of the leaves and severe vein distortion, were indistinguishable from those produced by the direct transfer of the virulent virus (pot A). Normal uninoculated beets are shown for comparison (pot D).

FIGURE 3.—Beet infected with virus attenuated by passage through *Chenopodium murale*

Figure 3 shows in detail the mild symptoms produced by the attenuated form of the virus. Only the younger leaves are affected, the symptoms being slight vein roughening and curling of the blade. The leaf petioles are of normal length, and the entire plant is almost normal in size. This beet is one of the four shown in Figure 2, B.

Figure 4 shows a test beet inoculated with the virus that had passed successively through *Chenopodium murale* and *Stellaria media* and shows in detail the symptoms produced by what is called, in this paper, the restored virus. The beets in Figures 3 and 4 were of the

same age and inoculated on the same date, seven weeks before they were photographed. The extreme shortening of the leaf petioles and general dwarfing of the entire plant, characteristic of the virulent virus, is shown in the plant inoculated with the virus from chickweed.

Several additional experiments were performed. The procedure was in general the same as that used in the preceding experiments. Table 2 gives the results of some of these experiments.

FIGURE 4.—A beet of the same age as the one shown in Figure 3 and inoculated at the same time but with virus that had been attenuated and later restored by passage through *Stellaria media*

TABLE 2.—*Results of inoculations of sugar beets with attenuated and virulent forms of curly-top virus before and after passing through Stellaria media*

[Ten leaf hoppers were fed on each test beet for three days]

Virus used	Beets inoculated	Beets infected		Type of symptoms
Before passage:	*Number*	*Number*	*Per cent*	
Virulent	20	26	89	Severe.
Attenuated	30	14	46	Very mild.
After passage:				
Virulent	10	10	100	Severe.
Attenuated	10	10	100	Do.

The results given in Table 2 show that the attenuated virus obtained from *Chenopodium murale* before passage through chickweed produced only very mild symptoms on the 14 beets infected. However, after passage through chickweed the symptoms were indistinguishable from those produced by the original virulent virus. The increase in the amount of infection from the virulent form of the virus after passage through chickweed is probably not significant because of the small number of beets used.

Another series of experiments (Table 3) were conducted over a period in which climatic and other environmental conditions varied a great deal, but the results of the various individual tests were practically of the same type and not at all contradictory. In series 1

the attenuated virus was obtained from stock cultures on susceptible sugar beets. These cultures were originally secured by transfers from *Chenopodium murale* to beets and were maintained by repeated transfers from beet to beet. Likewise the restored form of the virus which was originally obtained by transfers from *Stellaria media* to beets was in this series obtained from stock cultures maintained on susceptible beets. In series 2 the restored virus was taken directly from chickweed at the date of each inoculation. The attenuated virus was transferred directly from *Chenopodium murale* each time. The virulent virus in each series was obtained directly from beets infected with the stock virus.

TABLE 3.—*Results of inoculations of sugar-beets with virulent, attenuated, and restored forms of curly-top virus*

SERIES 1 [a]

Date of inoculation	Virulent form of virus			Attenuated form of virus			Restored form of virus		
	Beets inoculated	Beets infected	Type of symptoms	Beets inoculated	Beets infected	Type of symptoms	Beets inoculated	Beets infected	Type of symptoms
July 14				4	2	Mild	4	4	Severe.
July 16				4	1	do	12	4	Do.
July 17				8	6	do	12	5	Do.
July 22				8	2	do	12	10	Do.
July 25				6	1	do	5	3	Do.
July 26				4	3	do	4	4	Do.
July 29				10	2	do	5	4	Do.
Aug. 2				11	7	do	4	4	Do.
Aug. 4				6	3	do	6	4	Do.
Aug. 11				8	0		12	12	Do.
Aug. 16	4	4	Severe	8	1	Mild	7	7	Do.
Aug. 18				7	6	do	9	9	Do.
Aug. 20	14	13	Severe	13	2	do	6	6	Do.
Sept. 4				8	4	do	8	8	Do.
Sept. 5	7	2	Severe	6	2	do	6	6	Do.
Sept. 9	6	4	do	13	5	do	21	11	Do.
Sept. 12	7	6	do	33	6	do	37	29	Do.
Sept. 18	20	14	do	20	7	do	19	15	Do.
Sept. 20	18	16	do	15	2	do	19	14	Do.
Total	76	59		192	62		208	159	
Percentage		77.6			32.3			76.4	

SERIES 2 [b]

Sept. 29	8	5	Severe	8	2	Mild	20	16	Severe.
Sept. 30				26	1	do	30	22	Do.
Oct. 2	7	5	Severe	16	8	do	38	25	Do.
Oct. 3	8	8	do				28	14	Do.
Oct. 7	7	6	do	20	1	Mild	27	25	Do.
Oct. 11	12	11	do	12	1	do	26	19	Do.
Oct. 15	12	11	do	12	0		23	18	Do.
Total	54	46		94	13		192	139	
Percentage		85.2			13.8			72.4	

[a] All virus strains from "stock cultures" carried in sugar beets.
[b] Virulent virus from beet "stock cultures;" attenuated virus direct from Chenopodium; restored virus direct from Stellaria.

The point to be noted from these two series of inoculations is that the results were very similar whether the restored form of the virus was transferred directly from chickweed to beets or was maintained continuously on beets before being used in these inoculations. This indicates the degree of stability of virus condition obtained.

While there were differences in percentage of infection in the individual experiments comprising these two series (Table 3), the respective average amounts of infection produced by the three forms of the virus and the respective types of symptoms are similar in the two series.

Table 4 represents a summary of the inoculations performed in series 1 during September, 1930. The average incubation periods are representative of those found throughout these two series of inoculations.

TABLE 4.—*Summary of comparative tests performed in September, 1930, with the three forms of the virus*

[Inoculations were made by using two leaf hoppers to each test beet plant]

Kind of virus used	Beets inoculated	Beets infected		Average incubation period	Type of symptoms
	Number	*Number*	*Per cent*	*Days*	
Original (virulent)	65	42	64	9.5	Severe.
Attenuated	83	21	25	13.1	Very mild.
Restored	110	82	74	9.9	Severe.

As Table 4 indicates, the average incubation period of the original virulent virus is slightly shorter than that of the restored form, but the difference is probably not significant. By contrast, the incubation period of the attenuated form of the virus averaged considerably longer than for either of the other two forms, and this difference seemed to be consistently maintained. While in this series of inoculations the percentage of infection produced by the restored virus was somewhat greater than that produced by the virulent form, the average for all experiments was slightly less. It is not believed that the differences are significant.

Weighings were made of the entire plants while green, in order to get some measure of the effect of the three forms of virus on the size and development of the infected beets. Table 5 shows the total and average weights of a number of beets infected with virulent, attenuated, and restored forms of the virus, as compared with healthy normal beets of the same age. Forty-two plants were used in each lot.

TABLE 5.—*Comparisons of green weights of normal sugar-beet plants and of plants infected with original, attenuated, and restored forms of virus*

[Weighings made eight weeks after inoculation]

Kind of virus used	Beets weighed	Total weight	Average weight
	Number	*Grams*	*Grams*
Original (virulent)	42	138	3.3
Attenuated	42	407	9.7
Restored	42	153	3.6
Healthy checks	42	485	11.5

While the beets infected with the original virulent virus and the restored form are generally severely affected, occasionally among these there is a beet that appears to be somewhat mildly diseased. The

disease is not so mild as that produced by the attenuated virus nor so severe as that usually produced by the restored form. Tests were conducted to determine the condition of the virus in a beet infected with the restored virus which showed such apparent lessening of virulence in comparison with the standard reaction for the same virus. By means of nonviruliferous leaf hoppers the virus was transferred from the typical and atypical plants. Sixteen beets were inoculated with virus from the beet with the milder symptoms, 12 beets were inoculated with virus from the beet with severe symptoms, and 8 beets were inoculated as checks with virulent virus from a stock culture. In the first lot 14 of the 16 beets became infected; in the second, 10 of the 12, and in the third, 6 of the 8 beets became infected. The symptoms in all lots were about equally severe, but none resembled the symptoms of the mildly diseased beet used as a source of virus. Later, virus was again transferred from the two beets used in the test just described, sets of 11 young plants each being inoculated with the virus from each beet. All of these beets became severely diseased, with identical symptoms. These tests indicate that this apparent difference in symptoms may be attributable to an individual host response and is not a contradiction of the general findings.

DISCUSSION

While chickweed (*Stellaria media*) is the only plant tested extensively for restoration qualities, it is probably not an important factor in restoring virulence of the virus under natural conditions, because it grows in moist and shady places which are unfavorable to the leafhopper vector, *Eutettix tenellus*. This insect prefers dry and warm places in the semiarid regions. Alfileria (*Erodium cicutarium* L'Her.), however, which plays an important rôle in the overwintering of the virus, is a very important host for the leaf hoppers in California during the winter and early spring. Investigations are now in progress to determine its effect on the attenuated virus.

SUMMARY

Virulent virus of the sugar-beet curly-top disease has been attenuated by passing it through *Chenopodium murale*. Experimental evidence indicates that this attenuation is due to a change in the quality of the virus rather than to the quantity of virus involved. The attenuated virus remained stable even though passed through successive generations of very susceptible sugar beets.

The attenuated virus has been restored to its original virulence by passing it through *Stellaria media*. Virulent virus passed through *S. media* remained unchanged.

The incubation periods of the virulent and restored forms of the virus were practically of the same length, but the attenuated virus had a much longer incubation period.

The average weight per beet attained by the plants infected with the attenuated virus was almost three times that attained by beets inoculated with either the virulent or the restored forms of the virus.

Tests of the virus from a beet infected with the restored virus which showed somewhat milder symptoms than normal indicated

that the virus involved was as virulent as that in the companion beets that were infected with the same virus and showed the typical severe symptoms.

Stellaria media is probably of little importance in restoring the virulence of the virus under natural conditions.

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VITAMIN CONTENT OF THREE VARIETIES OF SPINACH [1]

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INTRODUCTION

Spinach (*Spinacia oleracea* L.) is recognized as one of our most valuable greens because it is an excellent source of vitamins and is available in the market all through the year. Since it is so extensively used many varieties have been developed, some of them differing materially in leaf shape, character, and color. Some varieties have smooth leaves, whereas the leaves of others are highly wrinkled or "savoyed." Leaf color varies from light yellowish green to dark green and bluish green.

The Bureau of Home Economics was interested in determining whether a relationship existed between leaf type or color and the content of vitamins A, B, and C. Accordingly, in 1928 a study was undertaken in cooperation with the Maryland Agricultural Experiment Station to determine the vitamin A, B, and C content of several selected varieties of fresh spinach.

For two years preceding this study the Maryland station had been growing different varieties of spinach to determine those best adapted to local conditions of cultivation. All of the spinach used by the Bureau of Home Economics in its tests was grown on the same plot of ground and during the same season.

REVIEW OF LITERATURE

VITAMIN A

The association of vitamin A with greenness of plant tissues has been demonstrated by several investigators. Dye, Medlock, and Crist (*2*)[2] have shown that the vitamin A content of lettuce varies more or less directly with the greenness of the leaves. Kramer, Boehm, and Williams (*4*) found that the green outer leaves of California head lettuce of the Iceberg variety were thirty or more times richer in vitamin A content than the whitest leaves from the centers of the same heads. Steenbock and Sell (*9*) made a chlorophyll analysis of etiolated leaves, entirely free from chlorophyll obtained from fresh heads of cabbage plants which at the end of the growing season had failed to head. The analysis showed that the white leaves contained about one-tenth as much pigment as the green leaves, which were found by animal feeding experiments to be superior in vitamin content. Collison and coworkers (*1*) found that the vitamin A activity of the unsaponifiable fraction from white cabbage was very small as compared with that of the corresponding fraction from the green leaves.

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[2] Reference is made by number (italic) to Literature Cited, p. 771.

Very little work has been done on the relation of leaf type to vitamin content. A study of the relation of vitamin A content to size of leaves of New Zealand spinach is reported by McLaughlin (*5*); she found that the vitamin A concentration of the leaf was directly related to its surface area and inversely proportional to its thickness. No other studies on the relation between content of vitamin A and leaf type were found.

VITAMIN B

The term "vitamin B" as used in this paper refers to the complex consisting of both the antineuritic and antipellagric factors. No record of a study has been found in regard to the variation of vitamin B content with greenness and leaf form.

VITAMIN C

No reference was found in the literature to the relation of antiscorbutic potency to leaf type or color.

DESCRIPTION OF VARIETIES OF SPINACH (SPINACIA OLERACEA)

The following descriptions of the three varieties of spinach which were used in this study are given by Geise and Farley (*3*): Variety, Virginia Savoy.—The plants are vase form, with leaves which are broad, thick, deeply wrinkled or savoyed, and dark green in color. Variety, Princess Juliana.—The plants grow very compact, with leaves heavily savoyed and bluish green in color. Variety, Viroflay.—The leaves are smooth or slightly crumpled near the base, spear shaped and thick, and are somewhat yellow green in color.

EXPERIMENTAL PROCEDURE

VITAMIN A DETERMINATIONS

The procedure for the determination of vitamin A was that outlined by Sherman and Munsell (*7*), with some modification. Young albino rats 28 or 29 days old were placed upon a basal diet devoid of vitamin A. It consisted of purified casein, 18 per cent; cornstarch, 67 per cent; dried brewers' yeast, 10 per cent; Osborne and Mendel salt mixture, 4 per cent; and table salt, 1 per cent. This was irradiated with rays of an ultra-violet lamp at 20 inches for one-half hour. After a preliminary period on this vitamin-A-free diet to deplete the bodily stores of vitamin A, the animals were placed in individual cages and fed graded weighed quantities of spinach six times a week in addition to the basal diet.

A litter of rats was used in such a way that when one rat of the litter was given a daily allotment of Virginia Savoy spinach, two others of the same sex and about the same weight were chosen, one to receive a like amount of Viroflay, and the other a like amount of Princess Juliana. Several litters, comprising a total of 82 animals, were used in this way, and conclusions were drawn from the average of the individual gains in weight. The spinach leaves were picked fresh for each feeding, and the feeding portions of each variety were all taken from similar parts of the leaves. If part of the stem of one variety had to be used, similar amounts of stem sections of the other varieties were used.

Figure 1 shows the average gain curves of groups of rats fed 0.025 g, 0.012 g, and 0.006 g portions, respectively, of the three varieties of spinach over a period of eight weeks. Sherman and Munsell have shown that the results obtained are more significant and more consistent when the rate of growth is about 3 g a week. Consequently, the relative vitamin A potencies of the three varieties of spinach are compared by determining the quantity of each which enabled the rats to gain at the rate of approximately 3 g a week. It will be noted that 0.012-g portions of Virginia Savoy, Viroflay, and Princess Juliana gave total gains in weight at the end of eight weeks of 22 g, 24 g, and 22 g, respectively. These results indicate that the three varieties are about equally potent in vitamin A.

This indication is further substantiated by the results obtained from feeding 0.025-g portions of each variety, all of which induced about equal growth rates. Furthermore, the total average gain in weight for the eight weeks of the groups that received 0.006-g portions, respectively, of the three varieties, was in each case 8 g.

VITAMIN B DETERMINATIONS

The determination of the vitamin B content of fresh spinach was begun before methods for testing for the two components of vitamin B were available. The method used was that of Sherman and Spohn (*8*), which makes no distinction between the two factors of vitamin B. All rats were kept in cages having raised screen bottoms and were fed a basal diet of purified casein, 18 per cent; cornstarch, 68 per cent; butterfat, 8 per cent; cod-liver oil, 2 per cent; Osborne and Mendel salt mixture, 4 per cent. In addition to the basal diet the animals were fed graded weighed portions of the three varieties of spinach six times a week.

The average gain curves are given in Figure 2. The groups of rats fed daily portions of 3 g of Virginia Savoy, Viroflay, and Princess Juliana had made total gains in weight of 40.5 g, 39.8 g, and 37.5 g, respectively, at the end of seven weeks (when the test period was terminated). These results indicate that the three samples of spinach contained about equal quantities of vitamin B. This conclusion is further justified by the results obtained when 2-g and 1-g portions were fed. The groups which received 2 g daily of Virginia Savoy, Viroflay, and Princess Juliana had made total gains in weight at the end of eight weeks of 9.5 g, 14.3 g, and 16.3 g, respectively. One gram a day of each sample of spinach just sufficed for body maintenance.

VITAMIN C DETERMINATIONS

The method used to determine the vitamin C content of spinach was that described by Sherman, LaMer, and Campbell (*6*). The basal diet consisted of heated skim-milk powder, 30 per cent; a mixture of equal parts rolled oats and bran, 59 per cent; butterfat, 10 per cent; and table salt, 1 per cent. In addition to the basal diet, a 1-g portion of each variety of spinach was given daily, six times a week. Since the supply of spinach was limited only a small number of animals were used, and the experimental period was terminated at the end of 77 days.

The results of the feeding tests with the spinach are given in Table 1. The two animals which were fed Virginia Savoy grew well and at autopsy showed no symptoms of scurvy. Of the 3 guinea pigs receiving Viroflay, 2 grew normally, and 1 did not thrive. It died on the seventy-sixth day and at autopsy showed mild symptoms of scurvy.

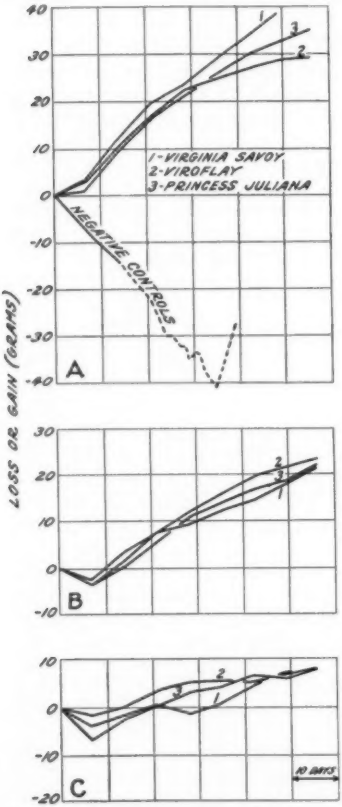

FIGURE 1.—Average changes in weight of groups of rats fed 0.025 g (A), 0.012 g (B), and 0.006 g (C) of fresh spinach as the sole source of vitamin A. In the case of the negative controls the broken line represents the average weight of the surviving animals until all had died

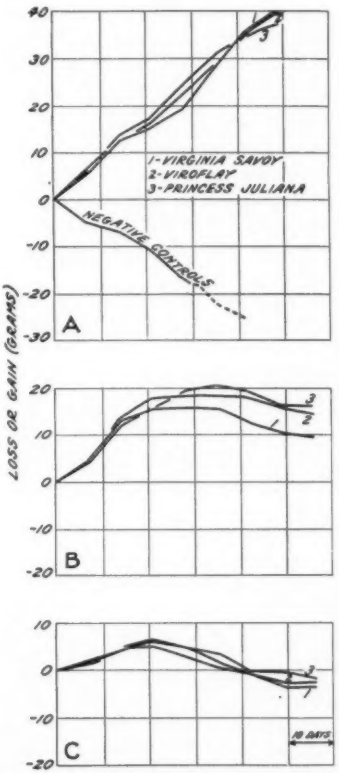

FIGURE 2.—Average change in weight of groups of rats fed 3 g (A), 2 g (B), and 1 g (C) of fresh spinach as the sole source of vitamin B complex. The broken line represents the average weight of the surviving animals until all had died

Three guinea pigs were fed Princess Juliana, and all gained in weight and lived to the end of the experimental period but on autopsy showed symptoms of scurvy. The data are insufficient to warrant definite indications, but it would seem that Princess Juliana was slightly less potent in vitamin C than the other two varieties tested.

TABLE 1.—*Results obtained when guinea pigs were fed 1-g portions daily (six times weekly) of three varieties of fresh spinach as the sole source of vitamin C*

No.	Variety of spinach used to supplement basal diet	Weight of animals during test period			Change in weight of animals during test period	Survival period	Degree of scurvy symptoms at autopsy
		Beginning	Maximum	Final			
		Grams	*Grams*	*Grams*	*Grams*	*Days*	
98 M	Virginia Savoy	342	676	676	+334	77	None.
99 M		353	702	702	+349	77	Do.
101 M	Viroflay	328	550	550	+222	77	Do.
102 M		360	806	806	+446	77	Do.
103 M		322	366	258	−64	76	None to mild.
104 M	Princess Juliana	373	690	667	+294	77	Do.
105 M		361	388	388	+27	77	Mild.
106 M		353	692	638	+285	77	Do.
107 M	Control	326	326	193	−133	20	Mild to severe.

SUMMARY AND CONCLUSIONS

Studies were made with three varieties of spinach to determine whether any correlation existed between vitamin potency and leaf type or leaf color.

The vitamin A content of the three varieties of spinach tested was about equal.

The three varieties of spinach seemed equally potent in the vitamin B complex.

The results indicate that Princess Juliana probably contained slightly less of vitamin C than the Virginia Savoy and Viroflay.

No relationship between leaf type and vitamin A or B content was detected. The variety with heavily savoyed, bluish-green leaves seemed slightly less potent in vitamin C than the other two varieties.

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CHEMICAL AND PHYSICAL PROPERTIES OF PETROLEUM SPRAY OILS[1]

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INTRODUCTION

Petroleum oils have long been employed as insecticides, but the injury that they cause, especially to the foliage of plants, constitutes a serious objection to their use. The literature of the subject has been reviewed by Kelley (*8, 9*),[2] Green and Johnson (*5*), and others.

Petroleum oils are highly complex substances. A complete analysis of a crude oil, showing all the individual compounds of which it is composed, has never been reported. The number of compounds in petroleum is very great, and their close similarity is the principal difficulty in separating and identifying them.

The analyses reported in this paper were made for the purpose of determining what properties of an oil may be used as a guide in estimating the injury that it will cause to plants.

METHODS OF ANALYSIS

For this work, a series of 13 samples of oil were chosen from a large number of spray oils that were available. An effort was made to select samples representative of the oils that were most injurious to plants, those that were least injurious, and a few that were intermediate. The most injurious oils are among those used as sprays on dormant trees and are not generally used on foliage.

The samples may be divided roughly into two classes, the light or highly refined oils, with a sulphonatable portion of less than 16 per cent, and the dark, or poorly refined oils, with a sulphonatable portion of more than 16 per cent. The term refined is used in this work to indicate the degree to which the materials soluble in sulphuric acid are removed, either by sulphuric acid or by liquid sulphur dioxide.

The method used to determine the sulphonatable portion is that recommended by Marshall (*12*). Twenty cubic centimeters of 37 N sulphuric acid is added in small portions to a 5 c c sample of oil in a Babcock cream-testing bottle, or an American Society of Testing Materials sulphonation bottle, and the bottle is immersed in an ice bath and shaken continuously until the temperature no longer rises. It is then put in a water bath at 100° C. and shaken at 10-minute intervals for 1 hour. At the end of that time sulphuric acid, having a specific gravity of 1 84 is added, until the clear unsulphonatable portion rises in the graduated neck of the flask. It is then centrifuged and the portion that has been sulphonated is calculated as the

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[2] Reference is made by number (italic) to Literature Cited, p. 786.

percentage of the original sample. With nearly all the samples, the method was found to give results that conformed closely to those furnished by the manufacturers.

Sulphur was determined by the oxygen-bomb method of Cross (*3*). The sample was completely oxidized in a bomb under 35 to 40 atmospheres pressure of oxygen and the sulphur subsequently determined in the residue as barium sulphate.

The determination of nitrogen was carried out according to the regular Kjeldahl method. A large sample (5.6 g) was taken and digestion was carried out slowly with the addition of successive portions of sulphuric acid until the oil had been completely oxidized and the solution was clear. Considerable care was taken at the beginning of the digestion in order not to cause too much frothing or volatilization of the oil. It was only with the dark, poorly refined oils that any trouble was encountered.

Acidity was measured by titrating a 10-g sample of oil in 50 c c of 95 per cent alcohol with 0.10 N alkali. The results were calculated to percentage of oleic acid, as suggested by Cross (*3*). The hydrogen-ion concentration was determined on the water extract prepared by shaking together for 1 hour 50 c c of oil and the same amount of water. A Bailey hydrogen electrode and a standard potentiometer were used to make the measurements.

The method of measuring bromine absorption was essentially that described by Scott (*14*) and Allen (*1*), which is a modification of the method proposed by McIlhiney (*10, 11*). Although bromine numbers are commonly determined on edible oils and drying oils, a satisfactory procedure has in this case been developed for petroleum oils.

Two-gram samples of oil were weighed into glass tubes of a convenient size, made by cutting off small test tubes about 1 cm in diameter. Each tube was then lowered into a clean dry 500 c c Erlenmeyer flask and 10 c c of carbon tetrachloride was added. Preliminary experiments showed that time and temperature affect the determination, so the reaction was carried out in a bath of melting ice and the time intervals were measured with a stop watch.

A short-stemmed separatory funnel, calibrated to deliver 25 c c portions, was fitted into the stopper of an Erlenmeyer flask. The funnel was filled with water, and a cylinder containing 70 c c of water and 5 c c of 60 per cent potassium iodide solution was set within easy reach.

An excess of approximate 0.33 N bromine-carbon tetrachloride solution was added to the sample in the Erlenmeyer flask. Usually 2 c c of the bromine solution was sufficient, but with the darker oils 4 c c was required. A large excess of bromine was avoided, as this would have influenced bromine absorption, as would also time and temperature. Immediately after the bromine was added, the separatory funnel, filled with water, was put in place and the stop watch was started. The flask was shaken for exactly 1 minute in the ice bath, after which 25 c c of water was added. After another one-half minute of shaking the 70 c c of water and 5 c c of potassium iodide solution were added. The addition of the last portion of water terminated the absorption of bromine by the oil. The remaining bromine was then titrated with standard 0.10 N sodium thiosulphate, starch being used as an indicator.

The aqueous portion contained the hydrobromic acid formed by bromine and the hydrogen liberated by bromine substitution. This

was separated from the oily layer of carbon tetrachloride and titrated with standard 0.10 N alkali. The indicator used was a methyl red-methylene blue proposed by Johnson and Green (*7*). A blank run was made containing all the reagents used in the determination. The difference in the amount of sodium thiosulphate used in the blank and that in the sample represented all the bromine absorbed by the oil.

Viscosity, flash point, and fire point were determined by standard methods used by the United States Government (*15*) for lubricating oils. Specific gravity, color, and emulsification with water were determined by the methods of Cross (*3*).

Color was measured against a standard solution containing 5 mg of iodine per 100 c c of water. The readings were taken in a Kleint colorimeter instead of by ordinary comparison tubes as suggested by Cross. The color of the iodine solution was taken as 1 and comparisons were made on this basis.

The values for emulsification are expressed in minutes required for a mixture of 27 c c of oil and 53 c c of water to separate after being stirred according to a strictly specified procedure.

The measurements of surface tension were made with a Du Nouy apparatus. A platinum wire loop exactly 4 cm in circumference was lowered beneath the film to be measured. The force required to pull the loop through the film was determined and expressed in dynes per centimeter of film. The emulsions used for surface film strength measurements were 4 per cent and 8 per cent cresoap, water, and oil emulsions prepared according to the formula for field emulsions of Melander, Spuler, and Green (*13*). The cresoap was also prepared from fish oil, potassium hydroxide, and cresol by the method of these writers.

Injury to plants was determined by applying the oils to the leaves of barley seedlings. The seedlings were grown in pure quartz sand and a nutrient solution of the following proportions was used.

240	c c of 1 molar KH_2PO_4.
192	c c of 1 molar $MgSO_4$.
192	c c of 1 molar $Ca(NO_3)_2$.
24	c c of 0.01 molar $FeCl_3$.
23, 352	c c of distilled water.
24, 000	c c total.

For each sample 12 barley kernels were planted in a small jar 10 by 14 cm. Extra jars were always planted so that those which were not up to standard might be discarded. In a light, warm room the seedlings would reach a height of 5 cm in about 7 days. They were then thinned to exactly 10 plants and the oil to be tested was applied carefully on both sides of the leaves with a camel's-hair brush. At the expiration of 3 to 5 days the treated seedlings were cut and weighed. At the same time control seedlings were cut and weighed. The percentage loss in weight of the treated plants, as compared with that of the control plants, that is, the difference in weight between the two, was used as the measure of injury.

Conditions for these experiments can not be exactly standardized, for practical reasons. With a more elaborate equipment of light, temperature, and humidity control, standard conditions might have

been more nearly approached. However, the method was quite successful as it was conducted, the results checking closely with those of duplicate determinations run at the same time.

An attempt was made to use bean plants, but because of the lack of uniformity in the plants it was discontinued. A few trials were made with pairs of bean leaves on the same plant, one leaf being treated and the other used as a control. This method is good when the degree of injury is determined by observation alone, but it is not satisfactory when more exact quantitative results are desired.

In order to find out whether or not the effect on barley seedlings would serve at least as an indication of the injury to be expected on apple leaves, a single series of determinations was made in the orchard. Each sample of oil was applied to both sides of 20 leaves on three different limbs, and at the expiration of 16 days the treated leaves were gathered and weighed. This experiment was conducted at a time when the leaves were making very rapid growth. The trial was carried on for too long a period, however, as some of the stronger oils completely killed the leaves, nevertheless the purpose of the experiment was fairly well accomplished.

EXPERIMENTAL RESULTS

INJURY

The method of determining oil injury by its effect on barley seedlings was developed after most of the analyses had been made. For a considerable time there has been a need for some standard with

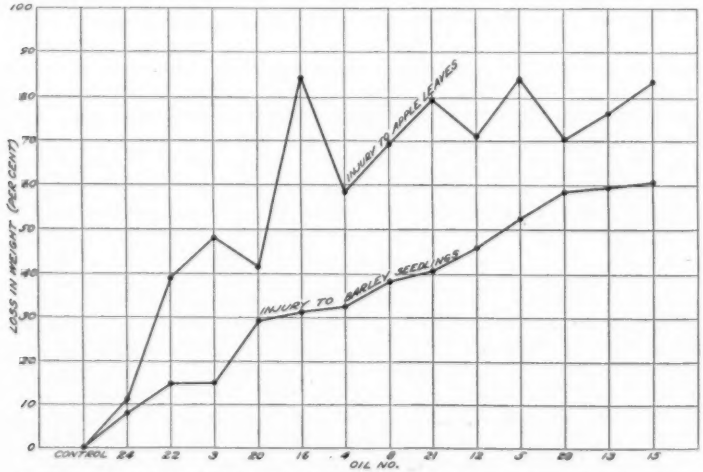

FIGURE 1.—Injury done to apple leaves and to barley seedlings when various oils were used for spraying

which the properties of the oils might be compared. Very early in the study Green and Johnson (*5*) attempted the measurement of oil injury by the changes in the respiration of plants. The changes proved to be too small and uncertain to measure by the methods then available, so the idea was abandoned. However, the results showed that the poorly refined oils caused an increase while the highly refined oils caused a decrease in the rate of respiration of bean leaves.

After developing the barley-seedling method for determining injury it was necessary to show that the results with this method were comparable to those obtained in the orchard. The data given in Table 1 and Figure 1 show the analyses of the oils and the relative injury that they caused to barley seedlings and apple leaves. In Figure 1 the oils are arranged in the order of increasing injury to barley seedlings. Oil No. 24, a colorless, highly refined oil with a paraffin base (the product of an eastern manufacturer) is the least injurious, while oil No. 15, a poorly refined, western oil, with a probable asphaltic base, is the most injurious.

TABLE 1.—*Analyses of spray oils and injury produced on barley seedlings and apple leaves*

Spray Oil No.	Analyses										Injury to—	
	Viscosity (Saybolt at 100° F.)	Flash point	Fire point	Color as compared with solution containing 5 mg iodine per 100 cc	Emulsification with water alone (minutes required for oil to separate)	Emulsification with water (cresoap as emulsifying agent)	Surface tension of pure oil	Surface tension of 4 per cent cresoap emulsion	Surface tension of 8 per cent cresoap emulsion	Specific gravity by Westphal balance	Barley seedlings as percentage loss in weight	Apple leaves as percentage loss in weight
		° F.	*° F.*				*Dynes per cm*	*Dynes per cm*	*Dynes per cm*			
3	113	310	345	(1)	0.5	(2)	35.4	40.5	35.4	0.873	14.7	47.8
4	53	280	295	0.20	5.0	(3)	34.6	36.1	35.1	.861	32.1	58.3
5	136	315	355	6.00	35.0	(3)	36.6	41.7	41.9	.919	52.5	83.8
8	425	360	410	33.33	6.0	(4)	37.0	42.8	39.2	.930	38.0	69.2
12	125	315	355	12.50	17.0	(3)	36.0	42.2	41.9	.908	45.6	70.9
13	82	300	340	3.60	6.0	(4)	36.3	38.4	38.6	.908	59.6	76.3
15	62	270	300	3.50	7.0	(3)	35.5	40.4	39.7	.896	60.7	83.9
16	56	280	310	.35	4.0	(4)	34.6	37.1	35.3	.874	31.0	83.9
20	98	330	360	.13	.5	(3)	34.8	38.1	36.6	.879	28.8	40.9
21	56	260	290	.37	3.0	(4)	35.1	37.6	35.3	.884	40.4	79.6
22	59	285	305	.25	.5	(3)	34.9	37.4	34.9	.863	14.4	38.9
24	68	350	390	(1)	.5	(3)	34.1	36.3	35.1	.837	7.8	11.0
28	110	325	375	8.50	9.0	(3)	35.1	41.9	39.7	.923	58.7	70.1

Spray oil No.	Analyses									Injury to—	
	Sulphonatable portion	Sulphur	Nitrogen	Bromine absorption	Bromine substitution	Bromine addition	Free fatty acids as percentage of oleic acid	CH× 10^{-4}	pH	Barley seedlings as percentage loss in weight	Apple leaves as percentage loss in weight
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*					
3	6.0	0.020	(5)	0.24	0.01	0.22	0.028	0.0136	5.866	14.7	47.8
4	12.0	.021	(5)	.89	.26	.37	.028	.0308	5.511	32.1	58.3
5	41.6	.290	(5)	5.73	2.76	.21	.170	.6420	4.193	52.5	83.8
8	53.0	.556	0.025	5.94	2.04	1.86	.170	.0320	5.495	38.0	69.2
12	27.5	.350	.030	5.48	2.28	.92	.085	.1460	4.835	45.6	70.9
13	31.2	.330	.012	4.93	1.62	1.69	.056	.2080	4.683	59.6	76.3
15	26.4	.213	.011	4.30	1.98	.24	.085	.5940	4.260	60.7	83.9
16	10.0	.110	.003	.98	.26	.46	.028	.4520	4.345	31.0	83.9
20	1.0	.041	.002	.52	.08	.36	.028	.0092	6.036	28.8	40.9
21	15.6	.042	.003	1.43	.42	.59	.042	.0455	5.342	40.4	79.6
22	2.0	.048	(5)	.24	.06	.12	.042	.0100	6.002	14.4	38.9
24	(5)	.042	(5)	.08	.02	.04	.014	.0116	5.934	7.8	11.0
28	35.2	.570	(7)	6.40	3.14	.26	.120	.1920	4.717	58.7	70.1

[1] No color, [2] Very poor. [3] Poor. [4] Good. [5] Very good. [6] None. [7] Trace.

The barley-seedling injury curve is the average of three determinations carried on at different times, while the curve for apple-leaf injury is the result of a single series of determinations, and hence is not so regular. With the exception of oil No. 16, the results are fairly comparable and indicate that the injury to barley seedlings is a fair measure of the injury to be expected on apple leaves.

As previously stated, some preliminary experiments were made on bean plants. Figure 2 shows two bean plants on which one of each of the pairs of leaves was treated with highly refined white oils, 3 and 24. The treated leaves appear little injured. Figure 3 shows leaves similarly treated with oils 5 and 28. Both of these oils are dark and poorly refined, and they caused very great injury.

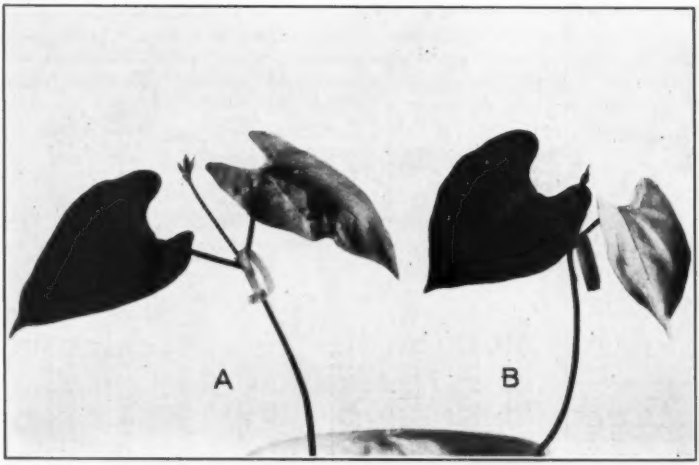

FIGURE 2.—Typical results obtained when white oils were applied to bean plants: A, Leaf with collar on its stem was treated with a highly refined oil, No. 24; B, leaf with collar on its stem was treated with a highly refined oil, No. 3

EMULSIFICATION

It is generally believed that the more easily an oil emulsifies the greater will be its injury to plants. Figure 4 shows a series of 8 per cent cresoap emulsions of the oils arranged in the approximate order of their ability to remain emulsified. These emulsions were all prepared in the same manner and were the samples used in the surface tension measurements. The more transparent tubes are the poorer emulsions and were made from oils 3, 24, 20, 4, and 22. They are among the less injurious. The tubes of more stable emulsions (on the left of fig. 4) are the less transparent, and were made from the injurious oils 28, 5, 12, and 15.

A more quantitative expression of the ease of emulsification of the oils is given in Table 1. Figure 5 shows that the oils which produce the most stable emulsions cause the greatest injury.

SURFACE FILM

In attempting to devise a method for the measurement of the covering capacity of the oils it was found that only those oils with a sulphonatable portion of more than 6 per cent would, of their

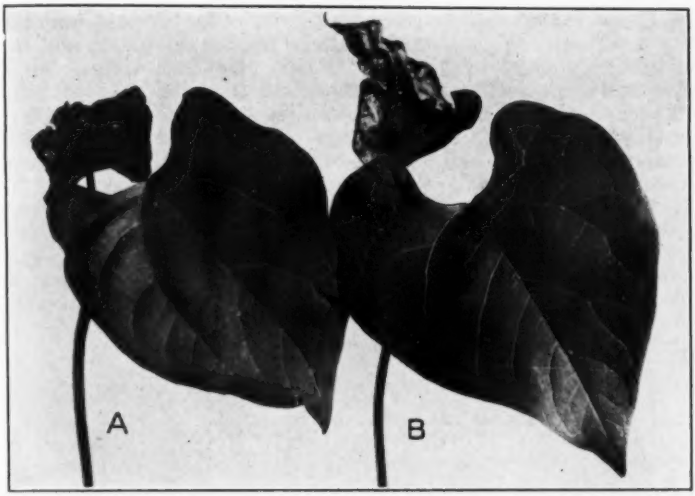

FIGURE 3.—Typical results obtained when dark poorly refined oils were applied to bean plants: A, The badly injured leaf was treated with oil No. 5, which had a sulphonatable portion of 46.1 per cent; B, the badly damaged leaf was treated with oil No. 28 which has a sulphonatable portion of 35.2 per cent

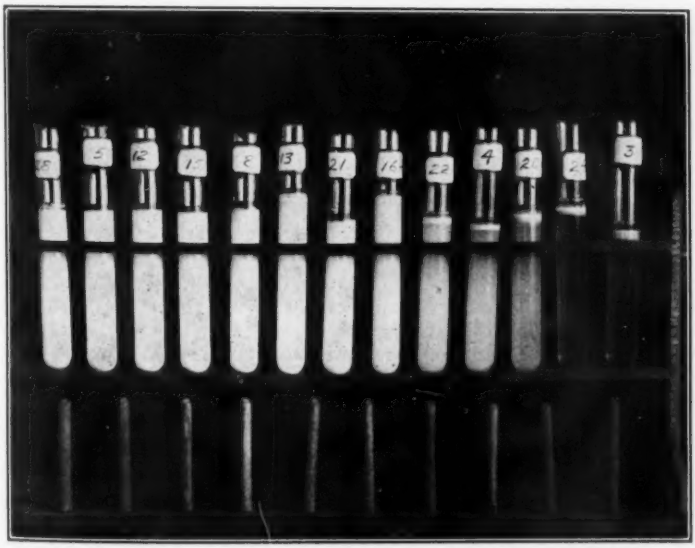

FIGURE 4.—A series of 8 per cent oil-water emulsions, emulsified with cresoap, arranged in the approximate order of increasing stability of emulsions

own accord, form a film on water. Oils 3, 20, 22, and 24, all with a sulphonatable portion of 6 per cent or less, would not spread on water, and these are among the least injurious. The oils with more than 6 per cent sulphonatable material spread on water, and these are generally more injurious. There is evidently a close relation between the degree of sulphonation and the ability of the oil to spread and also to emulsify. According to the Langmuir theory, the sulphonatable materials, or the materials which spread easily on water, are polar compounds, one portion of which is strongly attracted by water, while the opposite portion is repelled.

The attempts to measure the covering capacity of the oils were made with the object of finally determining the molecular size. After one film of oil had been applied to the surface of the water, the addition of more oil caused it to be driven into a smaller space with a piling up of the molecule to an unknown thickness. Figure

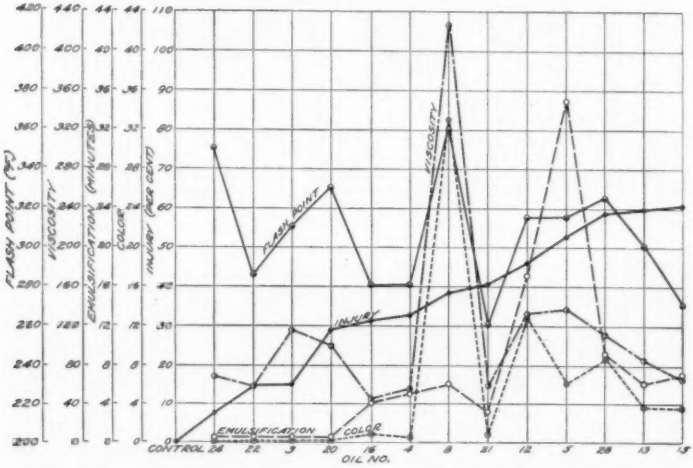

FIGURE 5.—Relation between physical properties of oils and their injurious effects upon barley seedlings when used as spray oils

6 shows the action of one film in driving aside another which had already been spread over the surface. The very dark portion around the outer edge of the watch glass, which is carbon black, was dispersed from the surface of the water by the spreading of the first film. The surface was then covered with a lighter colored dust (talc) and this was dispersed by applying more oil to the center of the surface. The process can be continued until the fourth or fifth film has driven the others to the outside. From these results it is apparent that the molecules of oil must be crowded until they are piled up as a polymolecular layer, the thickness of which is irregular and can not be measured.

SURFACE TENSION, COLOR, VISCOSITY, FLASH POINT, FIRE POINT, AND DENSITY

As may be seen in Table 1, the differences in surface tension are small. The pure oils vary from 34.1 dynes for oil No. 24 to 37.0 dynes for oil No. 8. If there is any trend, it is for higher surface

tension to be found in oils with greater amounts of sulphonatable materials, or those which cause greater injury. The surface tension of the emulsions parallels closely that of the pure oils, as would be expected.

The results of this investigation indicate that the color of an oil is related to the injury that the oil may cause to plants, The relation of color to injury parallels closely the relation of the sulphonatable portion to injury, and it is not known which of these variables is responsible for the damage to plants. Sulphonation, or treatment

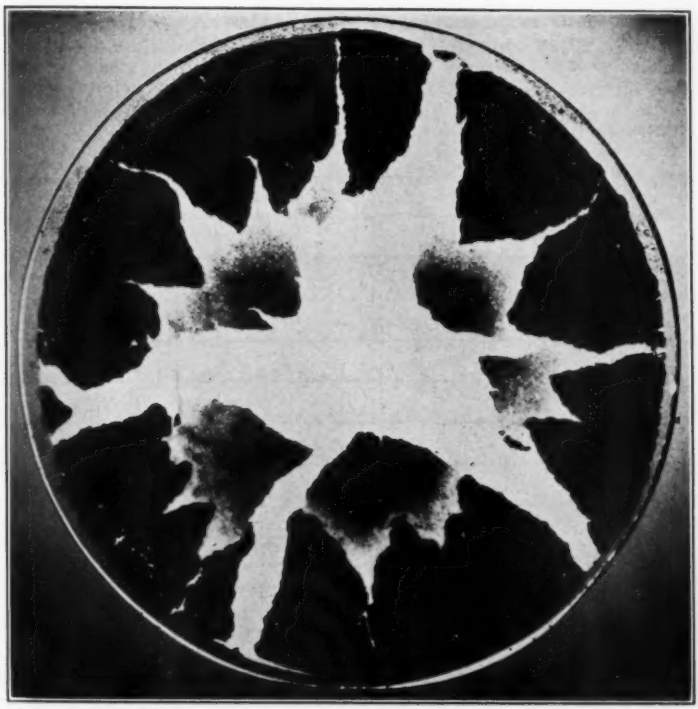

FIGURE 6.—Photograph demonstrating the ability of one oil film to interpose itself upon another. The black outer ring is carbon black dispersed by the first film. The lighter colored particles, covering largely the central portion, were spread upon the first film and were dispersed by the addition of more oil to the center of the surface

with sulphuric acid, removes the dark materials and, as Bell (*2*) has pointed out, they may also be removed by filtration, but no data are available on the effect of filtration.

Viscosity, flash point, fire point, and density seem to have little relation to the amount of injury caused by the oils. Viscosity receives a great deal of attention in spray practice, but, on the basis of the data presented here, it does not appear to be related to injury. The values for density show a slight general increase with increasing injury.

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SULPHONATION

The compounds removed from petroleum oils by sulphonation have been only approximately determined. It is obvious that the more reactive substances would unite with sulphuric acid. Gruse (*6, p. 121*), quotes Zaloziecki as giving the following outline for the reaction of sulphuric acid and petroleum:

> Sulfur compounds, resins and petroleum acids are dissolved or precipitated. Nitrogen bases and some unsaturated hydrocarbons are combined with the acid. Unsaturated hydrocarbons are polymerized. Some unstable hydrocarbons are oxidized. Aromatic hydrocarbons are sulfonated.

Undoubtedly sulphonation removes materials which cause injury to plants. Gray and De Ong (*4*) state that the sulphonation test is the best single criterion by which to determine whether or not a spray oil has objectionable properties. Their conclusions are confirmed by the results of this investigation. The greatest inconsistency in the correlation of injury with sulphonation is shown by oils

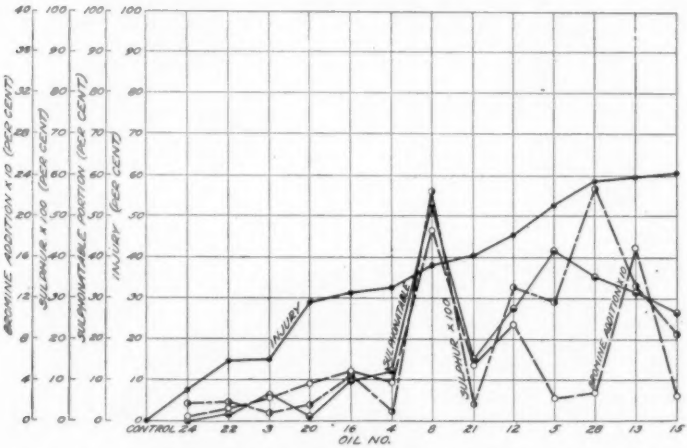
BROMINE ADDITION X 10 (PER CENT)
SULPHUR X 100 (PER CENT)
UNSULPHONATABLE PORTION (PER CENT)
INJURY (PER CENT)
INJURY
SULPHONATION
SULPHUR X 100
BROMINE ADDITION X 10
CONTROL 24 22 3 20 16 4 8 21 12 5 28 13 15
OIL NO.

FIGURE 7.—The relation of chemical properties of spray oils to injury of barley seedlings

13 and 15. These are the most injurious oils, but their sulphonatable portion is only 31.2 and 26.4 per cent, which is not so great as oils 5, 8, and 28.

BROMINE ABSORPTION

As sulphuric acid combines with the more reactive materials, bromine should do somewhat the same. From Figure 7, which presents the values for sulphonation and bromination, it is apparent that there is a relation between the two reactions. Bromine addition is a measure of the unsaturated, or multiple bonded, compounds. Bromine, being very active, easily combines at the point of multiple bonds and thus the compound becomes saturated. In other compounds where hydrogen atoms are held loosely, bromine easily takes the place of these poorly attached atoms and becomes fixed in the molecule by the process of substitution. The hydrogen liberated then unites with uncombined bromine, forming hydrobromic acid.

Every atomic weight of hydrogen displaced forms a molecular weight of hydrobromic acid. By titration of the hydrobromic acid it is thus possible to calculate the amount of bromine used in substitution. The compounds that take part in substitution are of the unstable class and may be active in plant injury.

The total bromine absorption, minus the bromine used for substitution, is expressed as bromine addition. If the unsaturated compounds of petroleum are ever proved to be the cause of plant injury, the measurement of bromine addition in spray oils will become an important phase of the analysis.

SULPHUR AND NITROGEN

Sulphur and nitrogen were present in comparatively small amounts in the oils examined. The maximum quantity of sulphur, 0.57 per cent, was found in oil No. 28 and the maximum quantity of nitrogen in oil No. 12. The fact that some of the very injurious oils, 5 and 28, contain only a trace of nitrogen, or none at all, tends to remove any suspicion that might be held regarding the injurious effects of nitrog-

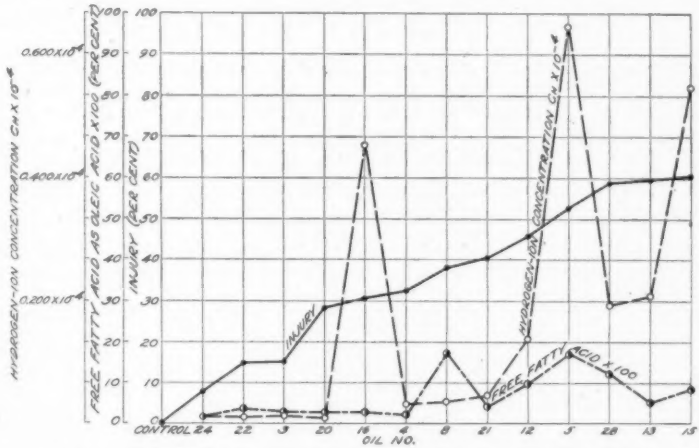
HYDROGEN-ION CONCENTRATION Cн X 10⁴
0.600X10⁻⁴
0.400X10⁻⁴
0.200X10⁻⁴
FREE FATTY ACID AS OLEIC ACID X100 (PERCENT)
INJURY (PER CENT)
INJURY
HYDROGEN-ION CONCENTRATION Cн X 10⁴
FREE FATTY ACID X100
CONTROL 24 22 3 20 16 4 8 21 12 5 28 13 15
OIL NO.

FIGURE 8.—The relation of the acidic properties of spray oils to injury of barley seedlings

enous compounds. The larger quantities of sulphur in the oils leave their part in plant injury still an open question.

ACIDITY

After the free acids had been run and the slight relation of these to injury noted (fig. 8), the hydrogen-ion concentration was determined. The four least injurious oils, 24, 22, 3, and 20, were found to have a very low hydrogen-ion concentration. From this point on the injury curve the acidity increased irregularly. Oils 16, 5, and 15, have an abnormally high hydrogen-ion concentration.

MODIFIED OILS

Because of some of the findings previously mentioned, experiments were conducted to improve the oils, and the search for the portion of the oil causing injury was continued. Since emulsification with

water had been shown to be related to injury, a small sample of the most injurious oil, No. 15, was subjected to long-continued washing with water. A 50 cc sample was put in a small percolating cylinder with an inverted siphon connected with the bottom. Water was allowed to drop into the cylinder, down through the floating layer of oil and out through the siphon. The falling water continually agitated the oil and carried out with it any soluble materials or compounds easily emulsified. The washing was continued for about eight hours and the oil was later tested on barley seedlings. As shown in Table 2, the normal oil caused an injury of 40.6 per cent, while the washed oil caused an even greater injury, 46.1 per cent. Tests were also made to determine the effect of complete sulphonation. Oils 5 and 15 were completely sulphonated according to the standard method. The improvement in oil No. 15 was very slight. An injury of 37.0 per cent was caused by the completely sulphonated oil No. 15 as compared with 40.6 per cent for the normal, while with oil No. 5 the injury decreased from 44.6 per cent for the normal to 24.6 per cent for the sulphonated. In the same series the completely sulphonated oil No. 24 caused an injury of only 12.8 per cent. These results indicate that sulphonation of spray oils is not a complete remedy for plant injury.

Oil No. 24 has been previously mentioned as an eastern oil with a paraffin base. Oil No. 3 is probably in the same class, but the other oils are from western fields. In view of the great difference in the degree of injury caused by completely sulphonated oils No. 24 and No. 15 it is probable that the location from which the crude oil was taken may have had an important bearing on the results. At least all injury can not be attributed to the sulphonatable portion.

TABLE 2.—*Loss in weight of barley seedlings during an interval of five days after spraying with oils Nos. 24, 15, and 5 which had previously undergone various treatments*

Oil No.	Treatment of oil	Loss in weight of barley seedlings (duplicate determinations)
		Per cent
24	Normal	12.8
	With 1.0 per cent ethyl mercaptan added	14.8
	With 0.05 per cent ethyl mercaptan added	24.9
	With 1.0 per cent ethyl sulphide added	15.2
	With 0.05 per cent ethyl sulphide added	19.7
	With 1.0 per cent amylene added	16.1
15	Normal	40.6
	Completely sulphonated	37.0
	Washed for eight hours with water	46.1
5	Normal	44.6
	Completely sulphonated	24.6

The data in Table 2 show the effect of adding two of the probable sulphur compounds of petroleum (ethyl sulphide and ethyl mercaptan) and one unsaturated hydrocarbon (amylene) to oil No. 24. In every case the injury was greater than that produced by the normal oil, but there is an apparent inconsistency with the sulphur compounds, in that the greater additions of sulphur caused the smallest increase in injury.

A series of experiments designed to change the acidity of an oil was made. Oil No. 15 was treated in three different ways. The first process was a distillation with steam for six hours in a strong sodium hydroxide solution followed by a thorough washing with water. The second process was a distillation with steam without alkali, and the third consisted in shaking a sample of the oil with an equal quantity of 50 per cent sodium hydroxide in a shaking machine. The treatments with alkali were intended to neutralize any acids present and the steam distillation without alkali would have driven off any easily volatile acids. The relative toxicity of the oils treated by the three processes was determined by their effect on barley seedlings, but no improvement in the oils was observed as the following data show:

Treatment of oil—Loss in weight of barley seedlings in five days (duplicate determinations)

	Per cent
Oil, with addition of an equal quantity of 50 per cent NaOH steam distilled for six hours, then washed thoroughly with water	65. 8
Oil steam distilled and not washed	72. 7
Oil, with addition of an equal quantity of 50 per cent NaOH, in shaker for five hours, then washed thoroughly with water	52. 2

Another series of trials was made with oil No. 15. Samples were treated separately with bromine, potassium permanganate, distilled with steam and sulphuric acid, shaken with soil, and the sample that was distilled with steam and alkali in the foregoing trials was washed with a large quantity of very dilute hydrochloric acid and then with water. The following results were obtained:

Treatment of oil—Loss in weight of barley seedlings in three days (duplicate determinations)

	Per cent
Shaken with $KMnO_4$, washed thoroughly with water, treated with potassium oxalate solution, and again washed thoroughly with water	20. 3
Shaken with excess of bromine, let stand overnight, and washed thoroughly with water	29. 7
Oil that was steam distilled with strong alkali (see tabulation above), washed with large quantity of 0.10 N HCl, and then washed thoroughly with water	30. 7
Distilled with steam and three times its volume of commercial H_2SO_4	12. 5
Shaken with an equal weight of clay soil, centrifuged, and washed	21. 1
Normal oil, no treatment	19. 5

The treatment with bromine should bring about complete saturation of the oil, while the action with potassium permanganate, although less definite, should tend to make the reactive compounds in the oil more stable. The distillation with steam and commercial sulphuric acid was only a modified sulphonation, and it will be noted that this was the only one of the processes that produced beneficial results.

The steam-alkali distilled sample brought forward from the work presented in first tabulation above was washed with dilute acid for the purpose of neutralizing any sodium salts of organic acids that might have been formed by the alkali treatment. The experiment was not successful however, for this sample was the most injurious of the group.

The purpose of shaking one sample with soil was to absorb some of the injurious fractions of the oil, but no significant result was obtained.

The data given in Table 2 and in the tabulations on p. 785 are from three tests with barley seedlings made at different seasons of the year and they are not therefore comparable. It is obvious that conditions of growth in tests made at different seasons will vary and that comparisons should be made only between samples that are grown side by side and at the same time.

SUMMARY

A laboratory method for determining the relative injury to barley seedlings caused by different oils and by oils treated by different processes, has been developed. The relation of the injury produced by these oils on barley seedlings in the laboratory to that produced on apple leaves in field experiments has been indicated.

Analyses of different spray oils have been made and the effect of the oils on barley seedlings is shown.

Although no single analysis of spray oils has been found to be directly correlated with injury, the relation of certain properties of the oils to injury is shown. Sulphonatable portion, sulphur, bromine absorption, acidity, ability to emulsify, and color all increase with increasing injury.

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BULK AS A FACTOR IN FORMULATING GRAIN MIXTURES FOR DAIRY CATTLE[1]

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INTRODUCTION

For many years treatises on the feeding of dairy cattle have stated that bulk is a factor that must be considered in making up a grain mixture for dairy cattle. A typical recommendation[2] reads as follows:

> Heavy feeds, such as cottonseed meal, ground corn, and feeds of that nature, if fed without being mixed with some bulkier feed tend to form a doughlike mass in the stomach when they become moistened and are not easily penetrated by the digestive juices. If, however, the grain ration contains a liberal portion of some bulky feed, as bran or feeds of that nature, it will remain porous when becoming moist in the stomach and will be easily digested. It is very important then that if a cow is being fed a large grain ration, that a liberal portion be of a bulky nature.
>
> Bran is probably one of the most common feeds used to give bulk to the grain ration. In addition it is a feed of high-protein content. Ground corn and cob meal is also often used in the dairy ration. The ground cob, while very low in feed value, has the distinctive value of giving the grain ration bulk. The fiber portions of ground grain sorghum heads are found to perform much the same purpose in that they add bulk.

This recommendation that the grain mixture should be bulky is not the result of experimental investigation. It is the result rather of the line of reasoning that if the grain mixture remains lumped together in the digestive tract, the digestive juices can not act upon it. However, account has not been taken of the fact that the rumen of the bovine is in a state of constant activity, and as a result the lumps or "boli" may be very effectively broken up and their contents mixed with the roughage part of the ration consumed by the animal. It is also possible that some of the boli are regurgitated and broken up by mastication.

Armsby[3] states:

> The rumen is so large that it always contains a considerable amount of material and the new feed when swallowed is more or less completely mixed with that already in the rumen by the peristaltic action of the latter, thus tending to prolong its stay. The liquid or comminuted portions probably pass on directly to the omasum, or manifolds, and the abomasum, but the bulk of the feed undergoes the process of rumination.

Colin[4], by means of a rumen fistula, observed the activity of the rumen and its ability to macerate and mix the newly arrived contents of the rumen and reticulum. He also expressed the opinion that finely pulped material may in part pass directly to the four reservoirs, or that that which does not pass immediately into the last two is soon washed there.

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[1] Received for publication Sept. 25, 1931; issued June, 1932. Journal Article No. 92 (new series) of the Michigan Agricultural Experiment Station.

[2] ANDERSON, E. E. FEEDING DAIRY COWS. N. Mex. Agr. Col. Ext. Circ. 94, p. 8, illus. 1927.

[3] ARMSBY, H. P. THE NUTRITION OF FARM ANIMALS. p. 82. New York. 1917.

[4] COLIN, G. TRAITÉ DE PHYSIOLOGIE COMPARÉE DES ANIMAUX, CONSIDERÉES DANS SES RAPPORTS AVEC LES SCIENCES NATURELLES, LA MÉDÉCINE, LA ZOOTECHNIE ET L'ÉCONOMIE RURALE. Ed. 3, enl., t. 1, p. 691-699. Paris. 1886.

Schalk and Amadon,[5] using the same method as Colin, reached a similar conclusion.

Nevens [6] fed ground corn, dyed with Congo red 4 B, to cows which were killed shortly after feeding. In every instance practically all the corn was found in the rumen and reticulum mixed with the other contents. Very little of the ground corn was localized, and in only one case was it found farther than the rumen and reticulum. He states: "The mixing had been done almost as thoroughly as could be done by hand or by means of a mechanical mixer." The findings of Nevens were therefore somewhat contrary to those of Colin [7], Schalk and Amadon, [8] and to the belief of Armsby [9] in that Nevens found practically all of the ground material in the rumen and reti ulum mixed with the other contents.

Before the investigation reported in this paper was begun, the results of an experiment carried on by the dairy section of the Michigan experiment station had shown that dairy cows receiving 14 pounds of linseed meal per day for several months, without the addition of bulky material, remained healthy and showed no tendency to go off feed. The quantity of linseed meal and corn consumed are given in Table 1. The results of this experiment suggested that it may not be necessary to consider bulk in making up a grain mixture for dairy cattle.

TABLE 1.—*Results of heavy consumption (pounds) of linseed meal and ground corn by cows during a long feeding experiment*

Animal No.	Lactation period	Average daily consumption from two to six months, inclusive, after calving [a]			Average daily consumption from calving to calving			Appetite
		Linseed meal	Corn	Total	Linseed meal	Corn	Total	
G-2	First	8.7	1.0	9.7	7.1	0.6	7.7	Good.
	Second	10.6	5.1	15.7	8.7	4.3	13.0	Do.
	Third	11.8	7.7	19.5				Do.
G-4	First	11.9	4.0	15.9	8.9	2.7	11.6	Do.
	Second	14.3	7.3	21.6	10.5	4.8	15.3	Do.
	Third	13.9	10.5	24.4	11.1	9.4	20.5	Off feed July 2–12, 1931. [b]
G-6	First	9.3	2.3	11.6	7.9	1.2	9.1	Good.
	Second	11.1	5.1	16.2	8.0	3.2	11.2	Do.
	Third	11.7	8.2	19.9	9.0	6.8	15.8	Do.
G-8	First	9.7	2.8	12.5	8.7	2.0	10.7	Do.
G-10	First	9.0	2.6	11.6	5.7	1.4	7.1	Do.
	Second	12.2	6.4	18.6	9.9	5.0	14.9	Do.
	Third	11.5	8.3	19.8				Do.
G-12	First	10.2	4.1	14.3	8.2	2.8	11.0	Do.
	Second	8.5	5.4	14.0				Off feed for about 50 days.
G-14	First	11.0	3.5	14.6	9.4	3.0	12.4	Good.
G-16	First	10.6	6.0	16.6				Off feed July 2–4, 1931.

[a] The average daily linseed-meal and ground-corn consumption from two to six months, inclusive, was taken because these animals were not placed on full feed for several weeks after calving, consequently food consumption for the first month was not as high as for the succeeding five months.

[b] Calved, June 29, 1931.

[5] SCHALK, A. F., and AMADON, R. S. PHYSIOLOGY OF THE RUMINANT STOMACH (BOVINE). STUDY OF THE DYNAMIC FACTORS. N. Dak. Agr. Expt. Sta. Bul. 216: 48–49. 1928.

[6] NEVENS, W. B. EFFECTS OF FASTING AND THE METHOD OF PREPARATION OF FEED UPON THE DIGESTIVE PROCESS IN DAIRY CATTLE. Jour. Agr. Research 36: 785–788. 1928.

[7] COLIN, G. Op. cit.

[8] SCHALK, A. F., and AMADON, R. S. Op. cit.

[9] ARMSBY, H. P. Op. cit.

The purpose of the present investigation was (1) to determine the ability of the rumen to break up and dissolve such lumps or boli as are formed during deglutition of a grain mixture, and (2) to find whether ground grains pass more or less quickly to the omasum and abomasum or whether they remain in the rumen and are mixed with its contents.

EXPERIMENTAL PROCEDURE

In this investigation 32 animals were used, one of which had a rumen fistula. The animals were given their regular feed of grain, silage, and hay about 6 a. m. About noon they were fed 5 pounds of a certain concentrate a certain number of hours before they were to be slaughtered, the purpose being to see how many boli could be recovered after certain feeds were given. Some of the animals were fed the concentrates before they were trucked to a slaughtering house, a distance of about 3 miles, and some were fed afterwards. Some were slaughtered locally and had to be led only about 100 yards. No animals were used that did not clean up the feed offered them within 15 minutes after it was fed. After an animal was slaughtered, the contents of the rumen were carefully sorted over by hand for lumps or boli present.

Linseed meal was used in a large number of these trials, since it is one of the most cohesive of cattle feeds when it becomes moistened. Five animals were fed 5 pounds of ground oats each and slaughtered one hour later. Five were fed 5 pounds of ground corn each and slaughtered one hour later. Three were fed a mixture of 2½ pounds of linseed meal and 2½ pounds of ground oats each and slaughtered one hour later. Three were fed 2½ pounds of linseed meal and 2½ pounds of ground corn each and slaughtered one hour later. Three were fed 5 pounds of linseed meal and slaughtered one hour later. Four were fed 5 pounds of linseed meal each and slaughtered three hours later, and five were fed 5 pounds of linseed meal each and slaughtered eight hours later.

The animal with a rumen fistula was used after the method of Schalk and Amadon.[10] In five trials she was fed 5 pounds of linseed meal one hour before the rumen was emptied through the rumen fistula, in five trials she was fed three hours before it was emptied, and in five trials she was fed eight hours before it was emptied. The rumen contents were replaced after they had been sorted over for boli, and the procedure was not repeated for at least 72 hours. A plug with a leather flange was kept in the opening of the fistula. This animal was in good condition after the experiments were finished, as shown in Figure 1.

In order to obtain information as to the course of the ground foods in the rumen and reticulum, three animals were given foods dyed with Sudan III and slaughtered as soon as possible after they had finished eating. Animal M236 was fed 5 pounds of a dyed grain mixture containing 3 parts ground corn, 1 part oats, and 2 parts linseed meal. This animal was slaughtered 15 minutes after she was given the feed. Animal M234 was fed 5 pounds of the same grain mixture that was fed M236, and slaughtered 20 minutes later. Animal 47 was fed 5 pounds of dyed ground corn and slaughtered 25 minutes later. The

[10] SCHALK, A. F., and AMADON, R. S. Op. cit.

feed of the five animals that received 5 pounds of ground oats and the five that received 5 pounds of ground corn and were slaughtered one hour after feeding, was dyed with Sudan III.

RESULTS

No boli were recovered from the rumen of the five animals that were fed 5 pounds of ground oats and slaughtered one hour later. The dyed oats were found fairly well distributed throughout the contents of the rumen. Likewise, no boli were found in the five animals that received 5 pounds of ground corn and were slaughtered one hour later; yet corn is generally considered a heavy feed.

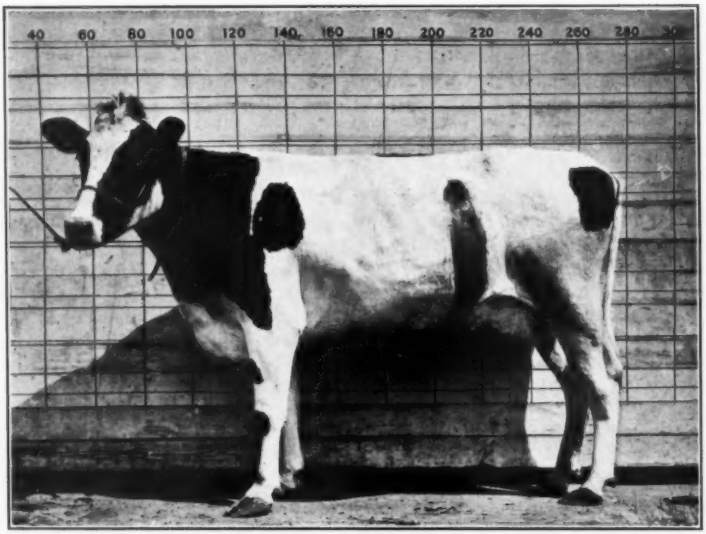

FIGURE 1.—Cow No. 238 with rumen fistula after the 15 trials reported in this investigation were finished. The plug has been removed in order to show the opening of the fistula.

The weights of the boli recovered from the various other ground feeds are shown in Tables 2 and 3.

When 2½ pounds of linseed meal was fed with 2½ pounds of ground oats and ground corn, respectively, the ground oats had the effect of separating the feed material, as is shown by the fact that much less of the mixture of ground oats and linseed meal was recovered than of ground corn and linseed meal. (Table 2.) However, a mixture of equal parts of ground corn and linseed meal would be about the heaviest and stickiest mixture that a farmer would ever use. In the three trials with this mixture only from 0.44 to 8.75 per cent of the concentrates was recovered in the form of boli when the animals were slaughtered one hour after feeding.

TABLE 2.—*Boli recovered from three animals slaughtered one hour after being fed 2½ pounds of linseed meal and 2½ pounds of ground oats, and from three others slaughtered one hour after being fed 2½ pounds of linseed meal and 2½ pounds of ground corn*

Animal No.*	Quantity of feeding recovered as boli	
	Grams	*Per cent*
ANIMALS FED LINSEED MEAL AND GROUND OATS		
204	3	0.13
C35	3	.13
171	6	.26
ANIMALS FED LINSEED MEAL AND GROUND CORN		
262	10	0.44
129	198.5	8.75
191	56.7	2.50

* All animals trucked to place of slaughter.

TABLE 3.—*Boli recovered from animals slaughtered 1, 3, and 8 hours after being fed 5 pounds of linseed meal*

Animal No.	Quantity of feed recovered as boli	
	Grams	*Per cent*
ANIMALS SLAUGHTERED ONE HOUR AFTER FEEDING		
26	113.4	5.00
223 *	453.6	20.00
256	510.3	22.50
ANIMALS SLAUGHTERED THREE HOURS AFTER FEEDING		
253	113.4	5.00
217 *	313.0	13.80
189 *	83.0	3.66
233 *	300.0	13.23
ANIMALS SLAUGHTERED EIGHT HOURS AFTER FEEDING		
231 *	1.0	0.04
M259 *	28.4	1.25
153 *	17.0	.75
184 *	12.0	.53
M266 *	5.0	.22

* Trucked to place of slaughter.

When 5 pounds of linseed meal was fed and the animals were slaughtered 1 hour, 3 hours, and 8 hours after feeding (Table 3), it was found that the rumen had considerable ability to break down and dissolve the boli. (Figs. 2 and 3.) When 5 pounds of linseed meal was fed and the animals were slaughtered one hour later the largest amount recovered was 22.5 per cent, but when the animals were slaughtered eight hours after feeding the largest amount re-

covered was 1.25 per cent. Very similar results were obtained with the animal having the rumen fistula. (Table 4.)

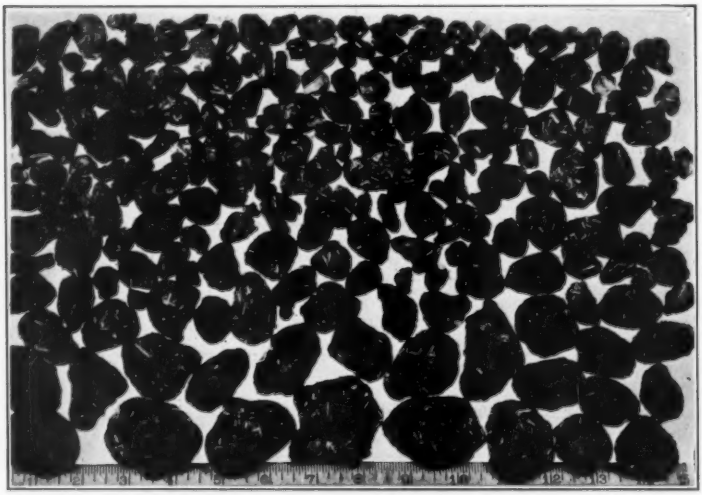

FIGURE 2.—Boli recovered from animal 256, fed 5 pounds of linseed meal and slaughtered one hour after feeding

FIGURE 3.—The approximate decrease of the boli of linseed meal in three (B) and eight (C) hours after feeding as compared to one hour (A) after feeding

By combining the results of the experiments in which linseed meal was fed and the animals slaughtered one hour later (Table 3) with the results of the experiments in which linseed meal was fed and the rumen emptied one hour later (Table 4), it will be found that the

average percentage of linseed meal recovered in the eight tests was 16.4. In the 9 tests in which the boli were recovered three hours later it was 8.2 per cent, and in the 10 tests in which boli were recovered eight hours later it was 0.6 per cent.

In the three trials in which the animals were slaughtered 15, 20, and 25 minutes after the dyed feed was offered, no feed was found in the omasum or abomasum. In the case of M234 and 47, which were slaughtered 20 and 25 minutes after being fed, the feed was fairly well mixed with the rumen contents.

TABLE 4.—*Boli recovered from animal No. 238, with rumen fistula, in 15 trials after feeding 5 pounds of linseed meal and having the rumen emptied 1, 3, and 8 hours after feeding*

Trial No.	1 hour after feeding		Trial No.	3 hours after feeding		Trial No.	8 hours after feeding	
	Grams	*Per cent*		*Grams*	*Per cent*		*Grams*	*Per cent*
1	611.0	26.94	6	21.0	0.93	11	17.0	0.75
2	323.0	14.24	7	160.0	7.05	12	9.0	.40
3	125.0	5.51	8	312.0	13.75	13	6.0	.26
4	562.0	24.78	9	121.5	5.36	14	18.0	.79
5	278.0	12.36	10	252.0	11.11	15	21.2	.94

In the tests in which five animals received 5 pounds of ground dyed oats and five received 5 pounds of ground dyed corn one hour before slaughter, there were two instances in which the dyed food was found in all four compartments. In one case the ground oats were found as far as the abomasum and in three cases as far as the omasum. However, the amounts found in both omasum and abomasum were insignificant.

DISCUSSION OF RESULTS

In analyzing the results obtained in this investigation it should be kept in mind that it is more than likely that the strength of contraction of the walls of the rumen varies somewhat in different animals, so that the material fed would be acted on more severely in some than in others. Moreover, the strength of contraction in the same animal may vary from time to time, as is shown by the varying results obtained from the animal with the rumen fistula. The hunger of the animals at the time of feeding would also influence the rate at which they would consume their food, and this in turn would affect the number and size of boli formed by deglutition. It should also be noted that there was as much variation in the weight of boli recovered from the animal with the rumen fistula as there was from the slaughtered animals. This would seem to eliminate any factor introduced by trucking the animals to the place of slaughter. In these studies no boli were noticed in either the omasum or the abomasum.

From the results of this investigation it would seem that the rumen has the ability to break up and dissolve boli even of the most cohesive feeds. The animals used in the tests mixed the feeds fed them with the roughage material of the rumen. Bulk therefore appears to be unnecessary in the grain mixture except possibly in the case of high-producing test cows that are being very heavily fed.

The results of this investigation indicate that the ground feeds all go to the rumen and reticulum and are mixed with the contents of these compartments. Once mixed with the rumen contents, they

await their turn at being exposed and washed to the omasum and abomasum. These observations concerning the course of ground feeds are in harmony with those obtained by Nevens [11] but not with those of Colin [12], and Schalk and Amadon [13]. The results of Colin and of Schalk and Amadon were obtained with animals having rumen fistulas, whereas those of Nevens and those of the present writers were obtained with slaughtered animals. Studies upon the course of feed similar to those of Colin, and of Schalk and Amadon were not made, since by their method it is necessary to remove a portion of the rumen contents, which might produce abnormal effects on the course of feeds.

Feeders often note that when heavy rations are fed, the animals show a greater tendency to go off feed than when light rations are fed. However, the results of the experiments herein reported seem to show that the reason for the cows going off feed may not be a lack of bulk in the grain mixture.

In order to obtain bulk in light grain mixtures farmers frequently purchase bran, although it is generally possible to obtain the quantity of protein found in bran in cheaper form in other high-protein concentrates. Such feeds as beet pulp, ground oat hulls, and ground alfalfa are sometimes added to ready-mixed feeds for the purpose of supplying bulk, and these cheap materials are thereby sold at grain prices.

In the light of the experiments herein reported it does not appear to be good practice for farmers who are feeding for economical milk production to purchase bran or any other feed to add bulk to the grain mixture when the protein of such feeds is more expensive than that in other protein concentrates. The placing of oat hulls, beet pulp, and ground alfalfa in commercial feeds for the purpose of adding bulk can not be justified. These feeds are primarily roughages and when they are placed in the grain ration they compete with the roughages produced on the farm.

SUMMARY

All the grains consumed appeared to pass directly to the rumen and reticulum, where they were mixed with the contents of the rumen.

When 5 pounds of linseed meal was fed and the animals were slaughtered one hour afterwards, from 5.0 to 22.5 per cent of the feed was recovered in the form of boli. However, when 5 pounds of linseed meal was fed and the animals were slightered eight hours afterwards only from 0.04 to 1.25 per cent of the feed was recovered in the form of boli.

The results obtained on an animal with a rumen fistula checked very closely with those obtained on the slaughtered animals.

Since boli are broken up and mixed with the roughage in the rumen and reticulum, it appears unnecessary to consider bulk in the make-up of a grain mixture for dairy cattle fed for economic milk production.

[11] NEVENS, W. B. Op. cit.
[12] COLIN, G. Op. cit.
[13] SCHALK, A. F., and AMADON, R. S. Op. cit.

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